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Experimental Study on Fixation of Allergic Reaction to the Eye.

By

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As is well known, the idea of allergy first suggested by Pirquet in 1906 has brought about a brilliant achievement in the recent development of pathology. In the background of this development, especially in the experimental field, there were two basic types of phenomena; that is, the so-called general and local anaphylaxis, or tissue-allergy. The latter was first described in 1903 by Arthus, so is usually known as the Arthus phenomenon. Because of its comparatively permanent findings in morphology this reaction was so adequate to experimentation on animals that a number of workers made an attempt to apply it in the same manner to the other different tissues and organs as the author previously did to the skin. Consequently, up to this time there have been reported many results concerning the allergic or, as Roessle has called it, hyperergic inflammation at the site of a local injection of antigen, for instance in the kidneys, joints, lungs, pleura, liver, appendix, genitals, muscles, tendons, arterial vascular system and so on. These investigations have indeed made a great contribution toward explaining the pathogenesis of several practical diseases, but it is also true that they do not elucidate the subject in full, for there is somewhere a gap between the occurrence of natural diseases in man and such an artificial process as direct injection of antigen into localized areas of animals. It may rather be said that these experiments were still within the category of the classic example designated by Arthus. So one of the experimental approaches to be next planned was considered in pursuing the possibility that the allergic tissue reaction might be produced under more natural conditions than mentioned above, and further studies have been made on the skin, muscles, pleura, joints and some arteries by several pathologists[1-4] and dermatologists, using non-antigenic physical stimuli such as heat, cold, mechanical or functional irritation, combined with an allergic mechanism.

On the other hand, in ophthalmology, besides the special kind of studies on the antigenicity of lens protein and of uveal pigment of the eye itself, there were reported, too, many studies on various ocular portions:

on the cornea, of which a typical one is noted as Wessely's phenomenon (Wessely, Poppen, Rhiem, Jirman, Ishida); on the uvea (Mazzei, Marchesani chesani, Rhiem, B.C. Seegal and D. Seegal, Brown, Rin, Moriji, Takahashi); on the conjunctiva (Rhiem, Hayashi, Funaishi); on Tenon's capsule (Isayama) and others (Spadavecchia, Nectoux). But these studies were as a rule carried out by local administration of foreign protein, for example foreign serum or egg white, into the cornea, anterior chamber, vitreous body, subconjunctival tissue, retrobulbar tissue or spaces between the sheaths of the optic nerve, Vv. vorticosae, A. carotis, or occasionally by instillation into the conjunctival sac. With regard to experimentation related to nonspecific stimulation, we do not find any other reports except the staphylococcic conjunctivitis or the traumatic uveitis in sensitive rabbits recorded by Rhiem,[5] the inoculation of certain bacteria or toxin into the anterior chamber recorded by Brown,[6] that of glycerin recorded by B.C. Seegal and D. Seegal,[7] and local administration of chloroform, histamine, uric acid and bouillonpeptone etc. recorded by Spadavecchia.[8]

The experiment here reported was carried out with the following purpose in mind: to determine whether the allergic tissue reaction can be localized upon the eyes of sensitive animals by means of local nonspecific stimulation, when it is done simultaneously with the second injection of antigen by intravenous route, and to study, if possible, the nature of ocular lesions in comparison with clinical cases.

At the present time, when the estimation of the allergy theory on clinical ophthalmic problems is not yet definitely understood and requires further critical study, it is, I believe, of great significance to bring about closer relations between experimental and clinical fields.

Experimental.

Methods and Materials.

The experiment was studied in the eyes of 35 rabbits, albino and a few mixed-breed, weighing from 1800 to 3000 gm., and as the antigen, sterile pig serum inactivated and occasionally cattle serum were supplied.

When the Arthus phenomenon was found to be positive after repeated subcutaneous or intracutaneous injections, commonly of 5 times, of small doses of the serum at the backskin, the following eliciting treatments were done locally on the right eye, the left eye being always untouched, of rabbits: Group I was treated with paracentesis and Group II with exposure of the corneal surface to heat of 45–50 C. thermophore or to cold of dry ice, for 5 to 15 minutes. The second injection was made immediately in every case in the marginal ear vein with a dose of 3,0 to 6,0 cc. of the same serum, and sometimes those methods were repeated. Few of the animals died

following the intravenous injection, but the surviving animals were all killed by air embolism at different stages after the clinical observation, at various interval of 1 to 40 days. As a control, some of the animals were put to the test without being previously sensitized.

Microscopic sections were made using 10 per cent formalin as a fixative, imbedding in paraffin or celloidin, and staining with hematoxylin-eosin, van Gieson's stain and, in case of need, with other special stains.

Results.

Group I: 15 albinos were put to use, 5 being retained as controls.

Clinical Observation: A few hours after the eliciting treatments, consisting of the second injection and the paracentesis, an acute irritative symptom of the eye appeared, reaching after 12 to 24 hours the highest manifestation with hypersecretion, lacrimation, swelling of the lids, chemosis, ciliary injection, and amounts of fibrinous exudate on the iris or in the anterior chamber, occasionally with effusion of blood into it. The iris became hyperaemic, edematous, and in a large proportion of cases a transparent dark-red shadow, which appeared to be bleeding in the area of ciliary processes, was discovered in the root-zone of the iris. The fundus was most frequently obscured by the haze of the anterior chamber. After a few days these irritations of the affected eye subsided relatively rapidly; the exudate and blood in the anterior chamber were considerably absorbed, remaining only in fine deposits on the pupillary border and the anterior lens capsule, and the shadow of bleeding on the back of the iris. In a few cases the reaction was less in degree; except for the mild hyperæmic symptom and the slight exudation in the anterior chamber none of the changes occured The opposite eye showed in general no response, but a few symptoms of increased lacrimation, chemosis or bulbar redness appeared in some of the rabbits.

Histologic Examination: Among the animals there were in fact several minor differences, partly due to the individuality of different rabbits and partly due to the various intervals of time observed, but the histologic findings as a whole can be summarized as follows: the cornea is almost intact except for a little cellular infiltration in the superficial tissue at the limbus. In the middle stages of reaction the anterior chamber is filled with a large number of erythrocytes and fibrin containing leucocytes, particularly in the angle. The stroma of the iris becomes loose or slightly edematous, in which the smaller vessels and capillaries are extremely congested, and in most of the animals extravasation of the erytheocytes is shown. In one rabbit which was killed at the later stages perivascular infiltrate of small round cells is shown. A slight but diffuse or rarely nodulous

form of infiltrate consisting of lymphocytic, polymorphonuclear, and less of plasma cells, is found in most animals mainly in the posterior layer of the iris (Fig. 1.). In the ciliary processes appears extensive edematous swelling, with varying sizes of Greefe's follicle and massive hemorrhage (Fig. 2., 3.).

Fig. 1.

Fig. 2.

In some animals the vessels are so intensively dilated and congested as to become static in appearance, and infrequently thrombosis with positive fibrin stain is shown. The fibrinoid homogenization is, however, in no case confirmed. The cellular reaction is more marked in the pars plana than in the ciliary processes and is present near the pigment cell layer with chiefly lymphocytes, polymorphonuclear, and less of plasma cells and histiocytes (Fig. 4.). In the posterior chamber the bleeding, with wander-

Fig. 3.

Fig. 4.

ing cells and fibrin, is found without an exception in company with the high changes of the ciliary body. In the vitreum too, adjacent to the retina, a few cells are seen apparently by migration in more than half the cases. Except for the engorgement of the retinal vessels with some of the surrounding leucocytes, appearing in a few sections, the retina as well as the lens

seems to be free from the present reaction. The choroidal vessels are in every portion considerably dilated, and filled with erythrocytes, leucocytes and blood plasma, especially near the ora serrata, with edema surrounding them. In addition, the choroid is infiltrated by the majority of the lymphocytes and polymorphonucleares with occasional mononucleares, diffuse with nodular accumulation, leading to a thickened tissue (Fig. 5.). In the sclera perivascular infiltration and vascular congestions were marked in some sections. Otherwise the optic nerve remained uninvolved. On the other hand, in the untouched eye sysmptom slighter than, but corresponding to those of the treated eye, such as swelling and bleeding in the ciliary processes, round cell infiltration in the pars plana, dilation and congestion of the vessels with cell infiltration in the choroid were sometimes seen.

In summary, the reaction here caused is an acute inflammatory one characterized mainly by the occurrence of a disturbance of circulation and cellular infiltration, particularly in the uveal tract. In contrast to these findings of sensitized animals, those of the control animals were remarkably less in degree, although there occurred edema in the ciliary processes, development of Greefe's follicle, hemorrhage in the posterior chamber and engorgement in the choroid.

Group II: This group was further divided into two groups by a kind of nonspecific stimulation; namely, 13 of the animals were exposed to heat, and 7 of them to cold, with 2 to 3 rabbits in each group being retained as controls.

Clinical Observation: In a few hours after the treatments the anterior segment of the eye became irritated, while on the second day there was increased swelling of the lida, hyperaemia and chemosis of the conjunctiva with hypersekretion and pericorneal injection, and occasional hemorrhage. The cornea, in addition to the epithelial changes, showed extensive edematous parenchymatous haziness, which increased until the visibility of the fundus was poor. In the iris there appeared hyperaemia, swelling and exudation, and in the anterior chamber massive serofibrinous exudate, frequently with hyphaema. These symptoms usually reached their height from the second to the fourth day, then decreased gradually. In the meantime the capillary loops at the corneosclerotic junction, which had been exposed to the local stimuli, became markedly red, extending inward as new-grouwth of vessels from the limbus to the centre of the cornea. Usually after 7 to 10 days the irritated symptom of the injured eye retrogressed, being healed with some corneal clouding. In respect to the corneal findings of these groups, exposure of the cornea to high or to low temperature brought certain different changes, but the findings of other ocular portions were similar in both groups. Some of the untouched eyes of the animals became also slightly irritated, as in Group I. On the other 2nd,

the control animals were not alike in the degree of their changes and the absence of the exudative reaction in the iris.

Histologic Examination: On the cornea there appeared patches of round cells in the subconjunctival areas of the limbus, deformity and desquamation of the epithelium, edema or swelling of the fibrous bundle in the substantia propria with leucocytous infiltrate, and corneal vascularization in the anterior layer; in the anterior chamber severe serous exudation with hemorrhage and migration of polymorphonuclear leucocytes; in the stroma of the iris engorgement and edema, occasionally infiltration of round cells or extravasation; in the ciliary processes remarkable dropsical swelling, congestion of the vessels with hemorrhage, and in the pars plana cellular reaction, the same as in the former Group. In addition, it seems noteworthy that the severest reactivity, quite extensive hemorrhagic and cellular reactions, was found in the corneosclearal region in one rabbit (Fig. 6.). In the choroid slight engorgement and a few infiltration cells appeared. The changes in the untouched eye, marked with disturbance of circulation and cellular reaction mainly in the vascular tissues, were distinctly milder than those in the treated eye.

Fig. 5.

Fig. 6.

In short, the chief lesions of this Group existed in the parenchymatous edematous changes of the cornea and the exudative hemorrhagic reaction of the iris and ciliary body; in other words, in the acute inflammatory reaction of several tissues surrounding the anterior chamber. In the control animals the corneal findings were almost similar to those of the sensitized animals, but the other tissues, for instance the iris or the ciliary body, did not become so inflamed.

Discussion.

As a result of the above-mentioned ophthalmoscopic and microscopic examination, it was primarily recognized that as for the intensity and ex-

tent of inflammatory reaction that occurred in the ocular tissue there was a distinct difference between the sensitized and the unsensitized animals.

But when we inquire analytically into the foregoing experimental conditions there appear at least two questions which should be considered in explanation of the pathologic findings presented. The first question concerns the tissue lesion, for which the local stimulation itself is responsible. Particularly, the corneal findings due to influences of the low or high temperature may be once left out of the problem of the "allergic" histopathologic features, for even in the control animals almost similar change of the cornea were found as well. However it is also to be kept in mind that in some of the highly sensitized animals abnormally high changes were presented in the corneoscleral region.

The second question consists in the influences of foreign protein that was used as the antigen. It was Iga[9] who gave a detailed account of this subject and who with v. Szily disputed an allergic basis of cellular infiltration in the ocular portion of the animals. Indeed it would be true that because of its rich vascular character the uvea is the site of the predilection of the toxic reaction, but in view of the relatively small dose of serum used in the formal experiment and the mildness of the changes in the control animals caused by the injection of the same dose as that of the second injection, we are obliged to hesitate in saying that the primary effect of the serum toxin is a causative factor of the experimental results.

Further, in view of the fact that certain changes in the opposite eye, which probably belong to the contralateral response recorded by Rhiem, Jahiel, Isayama, Moriji, Takahashi and others, were found exclusively in the sensitized animals and, moreover, have had a close resemblance to the histopathologic picture of the first eye, the impression gained is that the contralateral eye of the animal, if highly sensitized, would take part in the reaction on the onset of the inflammation of the first eye. The most that can be said here is that though the picture must be consequent, as Seegal stated, on the generalized hypersensitiveness, some unknown factors in connection with an action from the one eye to the other might play a rôle.

One of the standpoints to be discussed when we are going to define the allergic phenomenon has been a matter of the specific histologic picture advanced by Roessle and his school. In 1923 Gerlach[10] described thoroughly the tissue lesion at the site of serum injection, such as circulatory changes, edema or swelling of the connective tissue, leucocytic reaction and hemorrhage in highly sensitized cases; in short, the inflammatory reaction marked by hemorrhagic exudative character. Klinge[11] thereafter pointed out fibrinoid homogenization of the connective tissue, leucocytic-histiocytic infiltration, and in addition edematous or myxomatous swelling of the connective tissue, proliferating cellular reaction, formation

of nodules consisting of leucocytes and histiocytes, occasional occurrence of giant cells, participation of changes in the walls of vessels and formation of granuloma, etc. According to Masugi[12] the character of the tissue allergy lay in anaemic capillaritis with edematous or fibrinoid swelling of the connective tissue and swelling of the bundle of fibres, disturbance of circulation such as stasis and fibrin thrombosis in the capillaries and precapillaries, sometimes leading to necrosis of the tissue, and participation of arterial changes. In this way they noted especially the changes in the connective tissue although they were not prepared to consider it per se as conclusive evidence of the existence of an allergy.

My study failed to develop the fibrinoid homogenization of the connective tissue, the arterial changes and others, so I can not deny that in histopathology there is a difference between the picture presented and the so-called allergic-hyperergic one. But it is also necessary to note the special relationship of the eye structure in anatomy and the experimental proof of the fact that when antigen comes from a far distinct place via the blood stream, its morphology can possibly vary in various degrees and forms from the typical picture. But the hemorrhagic-exudative character as Gerlach called, and then the rapid and violent onset of the phenomenon in the clinical course, which is said to be one of the most characteristic signs of allergic inflammation, were surely demonstrated in our cases, too.

Recently some authors[13] are inclined to be of the opinion that the allergic nature of the phenomenon should be defined from another point of view, in other words from the angle of the mechanism of the onset instead of the resultant morphologic picture. Looking back on my experimental processes, the paracentesis, which causes an immediate drop in intraocular tension, engorgement and changed permeability of the vessels, and the changes of aqueous components etc., is one of the severest stimulations to the eye, so that if some substances such as antigenie ones simultaneously administered by way of the blood stream they would have reason to reach the eye rather than any other organ, and this fact seems to be applicable even to the following case where the local stimulation due to low or high temperature would bring engorgement or something irritative to the eye, though relatively limited to the anterior segment.

Therefore, it should be clear that my experimental treatment has led to the concentration of the antigen, the eliciting of an antigen-antibody reaction and then the fixation of reactive changes to the ocular tissue. And, as the changes obtained arose from the basis of sensitization, their essential nature should be after all attributed to the allergic mechanism, while the mechanical or physical stimuli should be regarded as a contributory factor in the development of that inffammation. In view of these results and this discussion, the possibility that a manifestation of

hypersensitiveness or allergic hyperergy can be fixed to the eye by means of the nonspecific stimulation, even if antigenic substances are not locally administered, has now been proved through the present work.

Conclusions.

The experimental work reported in this paper showed the possibility that an allergic ocular reaction can be produced not only in previously specifically sensitized eyes, but also in eyes that have been, under certain conditions, exposed to a nonspecific mechanical or physical stimulation. The experimental proof of the fact that the pathologic changes obtained have been partly owing to the antigen coming from a distant place via the blood stream suggests to us that there is a wider field of a hematogenous form for the pathogenesis of manifestation of ocular allergy.

References.

1) Knepper, Virchows Arch., 1935–36, **296**, 364.
2) Knepper and Waaler, Virchows Arch., 1934–35, **294**, 587.
3) Vaubel, Beitrag. path. Anat., 1932, **89**, 374.
4) Takeda and Shimpo, Transact. Soc. Path. Jap., 1935, **25**, 199.
5) Rhiem, Klin. Mbl. f. Augenhk. 1929, **83**, 336; Med. Klin., 1934, **30**, 1317.
6) Brown, Amer. J. Ophthalm., 1932, **15**, 19.
7) Seegal and Seegal, J. Immun. 1933, **25**, 221.
8) Spadavecchia, Zentralblatt f. d. gesamt. Ophthalm., 1938–39, **42**, 669.
9) Iga, Klin. Mbl. f. Augenhk., 1930, **84**, 449.
10) Gerlach, Virchows Arch., 1923, **247**, 294.
11) Klinge, Klin. Wschr., 1927, 2265.
12) Masugi, Transact. Soc. Path. Jap., 1939, **29**, 603.
13) Kitamura, Transact. Soc. Path. Jap., 1941, **31**, 515.

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Biochemical Studies on Carbohydrates.

CXXXIV. Mucosin.

First Report.

By

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Mucoitin (hyaluronic acid of Meyer) from umbilical cord was subjected to acid hydrolysis and amorphous mucosin given to esterification and acetylation. The final crystalline product proved to be heptacetyl-glucosamine-galacturonide-methylester (crystal water, $3H_2O$) by its behaviour toward Ehrlich reagent, analyses and molecular weight. It had the rotation of $[\alpha]_D^{10} = +14.8°$ in chloroform and F.P. of 130°C.

Experimental.

Mucoitin.

Umbilical mucin was prepared according to Masamune and Yasuoka[1] by precipitating the funis extract at pH 2.0. 8 g. of it were triturated in a mortar, dissolved in 500 cc. of water by the aid of the saturated $Ba(OH)_2$ and shaken with 5~10 cc. of formalin for 3~4 days, maintaining the pH of the mixture at 7~9 by occasional addition of the baryta solution. The centrifuged supernatant liquid (turbid) was adjusted to pH 7.0 when necessary, and after distillation in vacuo to 30~40 cc., precipitated with 2 volumes of alcohol (95%). The deposit was dried, after washing with alcohol and ether, in a desiccator. 4 g. of substance were obtained on an average. Biuret positive. This crude product was next taken up in 250 cc. of water, removed from the insoluble part by repeated centrifugations and subjected again to the formalin treatment similar to above (shaken with formalin for 2 days). Some precipitate was centrifuged off. The supernatant liquid was not transparent and still gave the positive biuret reaction. Therefore it was shaken with 40~55 cc. of a chloroform-amyl-alcohol mixture (2:1 by volume) for 5~6 hours at a pH of about 7.5 (brought to this pH by means of baryta) with following centrifugation. Another repetition of this operation gave a clear supernatant liquid that was biuret

negative. The liquid was dialyzed for 4~5 days and condensed by vacuum distillation to about 20 cc. and alcohol added. The precipitates were washed with alcohol and ether and dried. The product was non-colored powder. The mean crop 2 g. It gave strong positive reaction for hexuronic acid (Goldschmiedt) but the negative for ordinary sugar (Molisch).

Quantitative analytical results of one of the products are listed in Table I.

TABLE I.
Composition of a Preparation of Mucoitin.

Analysis	In per cent	In equivalents per equivalent weight
Nitrogen*	3.21	1.02
Glucosamine	39.96	1.00
Glucuronic acid		
By indole r.	42.68	
By orcinol r.	43.08	
Mean	42.88	0.99
Ash†	12.3	

* Micro Kjeldahl. † Micro analysis.

Muscoin.

3 g. of mucoitin were dissolved in 13 cc. of water and refluxed with 10 g. of oxalic acid at 100°C for 5 hours. The hydrolysate was cooled quickly and filtered. The bulk on the funnel was washed with several small portions of ice-cooled water and the filtrate and washings evaporated together on a boiling water-bath to a small volume (about 5 cc.). It was subsequently chilled, the precipitating oxalic acid separated off on a suction funnel and washed (5 cc. water used) and the filtrate and washing poured in drops into 150 cc. of absolute alcohol under agitation. The flocculent precipitates were centrifuged and washed with alcohol several times and with an alcohol-ether (1:1) mixture once and dried. About 1.5 g. of amorphous powder (crude mucosin oxalate) were given.

Free mucosin was separated by the aid of baryta:— 400 mg. of the above powder were taken up in 20 cc. of water, an insoluble part separated off and 158 mg. of crystalline barium hydroxide in 10 cc. of water added. Since baryta used was found insufficient, though calculated, to eliminate all oxalic acid, it was supplemented carefully and the centrifuged supernatant liquid (pH, 4.5) distilled in vacuo to dryness. The remainder was dissolved in about 10 cc. of water, filtered under suction and the filtrate again evaporated to dryness. Redissolution, filtration and re-evaporation was carried out more 3 times and finally to the condensate

(about 3 cc.) obtained by stopping evaporation halfway were added 25 cc. of absolute alcohol. And the precipitates were separated by centrifugation and washed with alcohol and ether. The yield of this portion was 150 mg. (Fr. I). From its mother liquid a second portion was thrown down by addition of ether (Fr. II). This weighed 100 mg. when dried. The former was reprecipitated from water (2 cc.) with alcohol (13 cc.) and after washing with alcohol and drying (crop, 80 mg.) subjected to analysis that gave the figures in Table II. The neutral equivalent was calculated at 381 on an ash-free basis (calc.: 355). The measurement here was processed as follows. 12.99 mg. of the substance were dissolved

TABLE II.

Composition of Mucosin as Percentages.

Analysis	Found		Calc. on ash-free basis
	Actual	On ash-free basis	
Nitrogen*	3.37	3.83	3.94
Amino nitrogen†	3.33	3.78	3.94
Glucosamine	43.01	48.88	50.42
Glucuronic acid			
By indole r.	50.17		
By orcinol r.	51.31		
Mean	50.74	57.67	54.64
Reduction as glucose of ferricyanide‡	45.86	52.11	50.70
Ash§	12.01		

* Micro Kjeldahl. † Micro Van Slyke. ‡ Analyzed according to Masamune[2] on the principle of Fujita and Iwatake. § Micro analysis.

in 2 cc. of water and titrated with 0.01 n. NaOH after addition of 1 cc. of twice diluted and neutralized formalin.[3] The alkali required was 3.901 cc. in the main run and 0.022 cc. in the control. In the test for hexosamine, the positive Elson-Morgan reaction was given but not the indirect Osaki-Turumi reaction. Molisch test resulted in negative.

Mucosinmethylester Hydrochloride.

0.4 g. lots of mucosin (prepared as Fr. I above) completely dried were dissolved in 2 cc. of absolute methanol involving 5% HCl and stood as stoppered at 20°C for 3 days. To the mixture were then added 10 cc. of absolute methanol and 20~30 cc. of dried ether and the flocculent precipitates centrifuged, washed with ether and placed in a desiccator. Yield, 0.32 g. Analyses of a preparation are embodied in Table III. Chloride test was positive.

TABLE III.

Composition of a Preparation of Mucosinmethylester Hydrochloride as Percentages.

Analysis	Found		Calc. on ash-free basis
	Actual	On ash-free basis	
Nitrogen*	2.81	3.20	3.46
Glucosamine	36.99	42.13	44.20
Glucuronic acid			
By indole r.	44.06		
By orcinol r.	44.91		
Mean	44.49	50.67	47.90
Acidity as hydrochloric acid†	7.39	8.42	9.0
Ash‡	12.2		

* Micro Kjeldahl. † Formol titration. ‡ Micro analysis.

Attempts were made to obtain a crystalline product. For this purpose crude mucosin oxalate (s. above) was immediately esterified and to the mixture after the reaction was added ether cautiously to faint turbidity. On standing crystals in needle form deposited but too small a yield did not allow analysis.

Heptacetylmucosin Methylester.

1 g. of amorphous mucosinmethylester-hydrochloride was put into a flask which was cooled in ice and a mixture of 6.7 g. pyridine and 5 g. acetic anhydride, similarly chilled, was added. The vessel was stood as stoppered for about 17 hours, with frequent shaking until complete dissolution of the powder. The reaction mixture was then poured into 30 cc. of ice-water under violent stirring and occurring amorphous precipitates and the mother liquid were separated by centrifugation.

a) The precipitates were taken up in a smallest possible quantity of absolute alcohol by boiling under reflux and the hot solution quickly filtered to eliminate the insoluble part. When cold grayish irregular crystals came out. Yield here was 250 mg. and F.P. 125°C. They were recrystallized in a like manner again and again to constant melting point of 130°C. The amount of the final product (non-colored needles) was 200 mg.—Prod. I.

b) The centrifuged supernatant liquid was distilled in vacuo (<40°C) and the residual dried as perfectly as possible in a desiccator for re-acetylation. The crystals first obtained (from alcohol) melted at 120°C. After recrystallizations the substance (needles)—Prod. II—melted constant at 130°C and no depression was shown by the mixed melting point test with Proid. I. Yield 50 mg.

The products (air dry) were united for examining the physical and chemical properties.

The substance dissolved in acetone, chloroform, ethylacetate, glacial acetic acid and pyridine with ease but in methanol, ethanol and benzene with difficulty. It was insoluble in ether, petroleum ether and cold water. It gave naphthoresorcinol (Neuberg-Saneyoshi), α-naphthol (Goldschmiedt) and aniline acetate tests for hexuronic acid and the indirect Osaki-Turumi test for acetylhexosamine. Hot Fehling and Tollens (cold) solutions were reduced. No ash was left on burning.

Crystal water.

6.040 mg. substance lost 0.469 mg. of water at 105°C under reduced pressure. $C_{12}H_{13}NO_{10}(CH_3CO)_7(OCH_3)+3H_2O$. Found 7.76%; calc. 7.53%.

Nitrogen (micro Kjeldahl).

13.20 mg. substance, 1.79 cc. 0.01 n. acid neutralized.

$C_{27}H_{43}NO_{21}$. Found 1.97%; calc. 1.95%.

Glucosamine (Hamasato and Akakura).

18.37mg. substance was taken for analysis. 2cc. of the hydrolysate, neutralized, filled up to 25 cc. and filtered, contained 0.443 mg. glucosamine as hydrochloride. Found 25.02%; calc. 24.97%.

Glucuronic acid.

17.60 mg. substance was dissolved in 10cc. of chloroform and 3cc. and 2.5cc. portions respectively were pipetted into test-tubes for indole and orcinol reaction with following drying. Then 1 cc. water was added for the colorimetric technique to each of the residues. Found 26.99% (by the indole reaction 26.70%, by the orcinol reaction 26.98%); calc. 27.06%.

CH_3CO (Suzuki's modification of Friedrich, Rapoport and Sternberg method).

10.620 mg. substance, 10.25 cc. of 0.01 n. thiosulfate solution used (4.4075 mg. CH_3CO). Found 41.50%; calc. 41.98%.

CH_3O.

3.626 mg. substance, 3.10 cc. 0.01 n. Na_2SO_3 used. Found 4.42%; cal. 4.32%.

Molecular weight from iodine value (Bergmann and Machemer[5]).

9.26 mg. substance, 3.249 mg. iodine used (iodine value 27.65). Found 723; calc. 717.

Optical rotation in chloroform.

$$[\alpha]_D^{10}=\frac{+0.45\times 100}{3.042\times 1}=+14.78^\circ \text{ (no mutarotation).}$$

The substance contained neither NH_2-N (micro Van Slyke) nor free acid group (by titration with alkali). For determination of NH_2-N 20.44 mg. substance was dissolved in 10 cc. of water by slight heating in a stoppered flask and 2 cc. of the solution subjected to analysis after cooling, whereby no crystals precipitated out within some time. Alkaline titration was made on 10.21 mg. substance in a mixture of 6 cc. water+6 cc. absolute alcohol. Alkali employed was 0.1 n.

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H. Masamune.

References and Notes.

1) Masamune and Yasuoka, Tohoku J. Exp. Med., 1947, **49**, 181.
2) Masamune and Tanabe, J. Bioch., 1933, **18**, 259.
3) Cf. a paper on chondrosin by Kobayasi (J. Bioch., 1944, **36**, 399).
4) Analyzed by Mr. Yosizawa according to Vieböck and Brecher.
5) Bergmann and Machemer, Ber. dtsch. chem. Ges., 1930, **63**, 1922.

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On Proteins and Amino Acids.

III. On Ascitic Fluid.

Third Report.

By

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A number of literature have accumulated regarding the serological specificity of colloidal substances in cancer tissues as well as body fluids of cancer patients. Nevertheless, Bermbach,[1] and Sunada and Komiyama[2] appear to have been the only investigators on ascitic fluid in this connection. Bermbach precipitated the serum of cancer patients with an antiserum produced by serum of a non-carcinomatous individual and added to the centrifuged supernatant liquid the serum immunized with carcinomatous ascitic fluid. The result did not give a definite evidence for the presence of specific proteins in blood of cancer patients. According to Sunada and Komiyama a "polysaccharide-like" substance is obtainable from the carcinomatous ascitic fluid and tissues of malignant tumors, which gives nearly specific precipitin and complement-binding reactions with sera of cancer patients.

In continuation of Aikawa's work[3] on ascitic fluid, the writers made a serological study of the two protein fractions in the fluid, non-precipitable (Prot. I) and precipitable (Prot. II) by electrodialysis.

Prot. I was not heat-coagulable in the absence of salt and did not lose solubleness in water on standing after washing with alcohol, although not only was its carbohydrate content minute but also it showed no remarkable distinction in colour reactions from ordinary simple proteins. Because of the property expedient for the purpose, this of the fractions from a carcinomatous ascitic fluid that originated in stomach cancer was chosen for producing an immune body. The obtained rabbit immune serum exhibited precipitin reaction with the antigen employed and Prot. II from the same source and further with corresponding preparations from any carcinomatous fluid no matter what the original seat and from non-carcinomatous fluids. Once adsorbed, however, with Prot. I from an ascitic fluid due to liver cirrhosis, the antiserum gave rise to evident precipitation

merely in cases Prot-I's from the carcinomatous fluids with primary stomach cancer were laid over it. The adsorbed serum was also inert to the extract of stomach cancers. It is not denied that the protein fraction, which was used as an antigen, was a mixture one of whose ingredients was responsible for the development of the antibody remaining after adsorption. So a part of its batch was subjected to fractional precipitation from water by various saturation with $(NH_4)_2SO_4$. But the subdivisions reacted with the adsorbed antiserum similarly and moreover closely resembled one another in nitrogen content and Molisch-positiveness, suggesting that the antigen consisted of a single protein. On the other hand, another part of the same batch was boiled with benzene, chloroform and ether in sequence and the extracts combined were distilled. The residue gave no precipitin reaction with the immune serum (unadsorbed) at all.

Whether the serological peculiarity of Prot. I from the cancerous ascitic fluids with primary stomach cancer is ascribed to the protein as a whole, to its amino acid grouping, to its carbohydrate moiety or to some contaminant is a question next to tbe answered. To begin with, a few enzymic, physical and chemical properties (digestibility, optical rotation, isoionic point, acid-binding force and amino index) of Prot.-I's were examined. But in so far as examined, no features discriminating the etiological nature of ascitic fluids were noticed. A further study is in progress along the same line. In passing, the cancerous Prot. I used for immunization exhibited precipitin reaction with serum of many patients with malignant tumor.

Experimental.

Preparation of the Protein Fractions from Ascitic Fluids.

An ascitic fluid was distilled in vacuo to about 1/10 the volume and dialyzed (a cellophane bag was used) against many changes of distilled water, until the reaction moved to neutral and thereafter the content of the bag (the precipitates and mother liquid together) electrodialyzed (a Pauli apparatus with collodion diaphragms, 200 volts) as long as water renewed in the outer compartments underwent the shift of hydrogen ion concentration (The electrodialysate in the anode compartment was distilled under diminished pressure to dryness (Fr. E) and that in the cathode compartment discarded). Voluminous precipitates in the middle compartment were filtered on a suction funnel and dried in a vacuum desiccator (H_2SO_4) —Prot. II. The filtrate (slightly acid) was distilled and the remaining syrup also dried similarly—Prot. I.

The properties of the ascitic fluids taken as starting materials and the yields of the protein fractions are listed in Table I.

TABLE I.

No. of ascitic fluid	Diagnosis	Colour and transparency	Specific weight	Relative viscosity	Protein content (Esbach) %	Rivalta	Cell	Volume cc.	pH	Yield (g.)			
										Fr. D	Fr. E	Prot. I	Prot. II
1	Stomach cancer, carcinomatous peritonitis	yellow, turbid	1018	1.34	4.4	−	erythrocyt., leucocyt., cancer cells	850	8.8	5.5	0.42	14	18
2	Biliary liver cirrhosis	yellowish brown, transparent	1014		2.8	−		300	8.9	2.5	0.05	0.85	1.2
3	Hepatoma, carc. perit.	hemorrhagic, centrifuged supern. liq.: yellow and turb.	1018	1.35	3.9–4.0	+	cancer cells	2,800	8.2–8.4		0.64	24	>38
4	Tuberculous perit.	light yellow	1022	1.88	8.6	+	lymphocyt., leucocyt., few erythrocyt.	1,300	8.0–8.2		0.28	>6	>8
5	Stom. canc., metastasis in choledochus, carc. perit.	hemor.	1018	1.38	3.8	+	many cancer cells, leucocyt., erythrocyt.	6,500	8.6–8.8		1.20	>32	>31
6	Stom. canc., carc. perit.	hemor., slight hemolysis	1018	1.50	4.6–4.8	+	many cancer cells, many erythrocyt., few lymphocyt.	2,900	8.8		0.72	>16	>20
7	Choled. canc., carc. perit.	yellow, translucent	1015	1.09	2.8	±	cancer cells	2,800	8.8	12	0.62	>10	24
8	Stom. canc., carc. perit.	very turbid	1015	1.16	5.2	+	many cancer cells, many leucocyt., erythrocyt.	3,800	8.8		0.98	58	74
9	Cardiac decompensation	light yellow, transp.	1012	1.02		−	few leucocyt., few lymphocyt.	400	7.8		0.06	>1.2	>1.9
10	Stom. canc., carc. perit.	hemor.	1018	1.81	7.6	+		2,500	8.2		0.58	>10	>15

Fr. E was set aside for isolation of the poison[4] whose description will be postponed to a future paper.

General Properties of Prot.-I's.

Prot.-I's were purified by a repetition of electrodialysis to completely remove water-insoluble Prot. II and precipitation with alcohol.

The purified preparations were colored light yellow or light brown. They were neither coagulated by heating in acidulated (with acetic acid) water nor denatured by treatment with cold alcohol. Precipitation of Prot. I of ascites No. 1 by $(NH_4)_4SO_2$ began at 1/4 saturation of the salt and was most evident at the full saturation. It was abundantly cast down by trichloracetic, sulfosalicylic and picric (both in the presence and absence of HCl) acid. It showed positive reactions for tryptophane (Hopkins-Cole; Kobayasi vanillin test[5]), histidine (Ehrlich diazo test), tyrosine (Millon; xanthoprotein test) and ordinary sugar (Molisch). Reduced sulfur test, negative. Indirect Osaki-Turumi test resulted in negative despite of the positive direct. Phosphorus, purine and bile acid (Pettenkofer[6]) were detected, but were so scarce as practically nil. Gmelin negative. Quantitative analysis gave 15.24% N (micro Kjeldahl), 0.8% hexosamine, 0.03% ash.

Other Prot.-I's possessed similar qualitative and quantitative (Their nitrogen content is given below) properties, irrespective whether an ascitic fluid which was fractioned was carcinomatous and possessing the primary seat in stomach or carcinomatous but possessing the primary seat in another organ or non-carcinomatous.

Properties of Prot.-II's.

They were not further purified.

They were insoluble in water, difficultly soluble in dilute acids (pH 2–1) and dilute alkalis (pH 8–9) but almost completely soluble in 10% hydrochloric acid and 1 n. sodium hydroxide. Osaki-Turumi test: the indirect, negative; the direct, positive. The positive Molisch reaction was given. The presence of phosphorus and purines was demonstrated. N, 14.5–14.8%; hexosamine, 0.7–0.8%; ash, 0.07–0.09%.

Production of an Immune Serum of Prot. I from the Ascitic Fluid No. 1 (Carcinomatous Peritonitis Originating in Stomach).

The purified product above of this Prot. I was intravenously injected to a rabbit weighing 3280 g. every four days. At the first and second time were applied 100 mg. of the substance as dissolved in 1.5 cc. of physiological saline and later 150 mg. in 2.5 cc. of the saline. Blood was shed after 5

days from the fifth injection and the serum separated. And 1/9 the volume of 5% carbolic acid was added for preservation (in an ice chest). It caused precipitation of the antigen (1%) at a dilution of 1:180.

This serum (undiluted) showed precipitin reaction (ring test) not only with the antigen employed for the immunization but also with Prot. II from the same source and the protein fractions from other ascitic fluids though not identical regarding sensitivity (Table II).

TABLE II.

Occurrence of Precipitation of the Ascitic Fluid Fractions, Prot.-I's Prot.-II's, by an Immune Serum of Rabbit Injected with Prot. I of a Carcinomtous Ascitic Fluid Originating in Stomach (Ascitic Fluid No. 1).

No. of ascitic fluid	No. of protein fraction	Dilution of protein solutions*					
		1% (Original)	×10	$\times 10^2$	$\times 10^3$	$\times 10^4$	$\times 10^5$
1	I	++	+	+	+	+	−
	II	++	+	+	+	−	−
2	I	++	+	+	+	−	−
	II	++	+	+	+	−	−
3	I	++	++	+	+	+	−
	II	++	+	+	+	−	−
4	I	++	++	+	+	+	−
	II	++	+	+	+	−	−
5	I	++	+	+	+	±	−
	II	++	+	+	±	−	−
6	I	++	++	+	+	+	−
	II	++	+	+	±	−	−
7	I	++	++	+	+	±	−
	II	++	+	+	+	−	−
8	I	++	+	+	+	±	−
	II	++	+	+	+	−	−
9	I	++	+	+	+	−	−
	II	++	+	+	−	−	−
10	I	++	++	+	+	+	−
	II	++	+	+	+	+	−

*The proteins were taken up in physiological saline. In the cases of Prot.-II's dilute NaOH and a NaCl solution were used for the dissolution.

In the next the antiserum was treated with purified Prot. I from the ascitic fluid No. 2 (non-carcinomatous, s. Table I). To 1 cc. of the former were added 15 mg. of the latter and after shaking, the mixture was stood for 1 hour in air and centrifuged. Adsorption was perfect after another similar treatment of the supernatant liquid. With this adsorbed serum the results in Table III were given.

The positive precipitin reaction (dilution of the protein fractions: 1 to 100–1000) did occur only with the protein fractions from carcinomatous

TABLE III.

Occurrence of Precipitation of the Protein Fractions of Ascitic Fluids by the Antiserum of Prot. I No. 1 Adsorbed with Prot. I No. 2.

No. of ascitic fluid	No. of Protein fraction	Dilution of protein solutions*			
		1% (Original)	×10	$\times 10^2$	$\times 10^3$
1	I	+	+	−	−
	II	±	−	−	−
2	I	−	−	−	−
	II	−	−	−	−
3	I	−	−	−	−
	II	−	−	−	−
4	I	−	−	−	−
	II	−	−	−	−
5	I	+	+	−	−
	II	±	−	−	−
6	I	+	+	−	−
	II	+	−	−	−
7	I	−	−	−	−
	II	−	−	−	−
8	I	+	+	−	−
	II	+	−	−	−
9	I	−	−	−	−
	II	−	−	−	−
10	I	+	+	−	−
	II	+	−	−	−

* The proteins were taken up in phsiological saline. In the cases of Prot.-II's dilute NaOH and a NaCl solution were used for the dissolution.

fluid having the primary seat in stomach, and of these the Prot-I's proved more reactive than the Prot.-II's. Contrary to expectation the unadsorbed antibody appeared to have no bearing on soluble proteins in the tissue of stomach cancer, because, when five fresh stomach cancers were ground with physiological saline (1 g.: 1 cc.), four of the extracts (separated by centrifugation, turbid) entirely did not and the remaining one did react but hardly with it. It was suspected that Prot. I used for the immunisation was a mixture of proteins one of which was responsible for development of the specific antibody. Therefore further fractionation of it by means of $(NH_4)_2SO_4$ was tried. With the aim in view 380 mg. of the preparation were dissolved in 14 cc. of water and fractionally precipitated by using at first the saturated salt solution and followingly with solid salt. The successive precipitates were stood for half an hour, centrifuged and washed with a solution of the salt equal in concentration to the mother liquid. They were then dialyzed against running water after dissolving in 10 cc. of water to eliminate the salt and finally distilled in vacuo. The recovered portions showed no discernible divergency from one another regarding the

serological reactivity to the adsorbed serum as well as composition (Table IV).

TABLE IV.

Composition and Precipitin Reaction of Portions of Purified Prot. I No. 1 after Fractional Precipitation with Ammonium Sulfate.

Precipitin test was exerted with the antiserum of the above protein adsorbed with Prot. I. No. 2.

Fraction	Saturation grade of $(NH_4)_2SO_4$ for precipitation (temp., 15°C)	Yield mg.	Nitrogen %	Molisch test	Occurrence of reaction at dilutions	
					$1:10^2$	$1:10^3$
Fr_1	3/10	20	15.6	+	+	+
Fr_2	4/10	40	15.5	+	+	+
Fr_3	5/10	35	15.2	+	+	±
Fr_4	8/10	240	15.3	+	+	±
Fr_5	10/10	15	15.3	+	+	+
Fr_6	Unprecipitated at the full sat.	18	15.1	+	+	−

They left practically no ash on burning.

The Lipoid Contaminants of Purified Prot. I No. 1 did not Give Precipitin Reaction with the Antiserum of the Latter.

1 g. of Prot. I No. 1 (purified) was powdered and boiled under reflux with 60 cc. of benzene (on a heated water-bath) for 30 minutes. While hot the mixture was centrifuged and the extraction repeated once more with same solvent. Like operations were further effected with chloroform, absolute alcohol and ether by turns. Then all the extracts were combined and distilled in vacuo and dried up in a desiccator. The substance obtained (a trace) was emulsified in 0.5 cc. of 0.02 n. Na_2CO_3 and 0.5 cc. of 1.8% NaCl added. The turbid solution and its centrifuged supernatant liquid were laid over the antiserum (unadsorbed) above. Precipitation did not occur at all, while, in the control run, the solution of the protein before the exhaustion gave distinct reaction as before.

Precipitin Reaction of Prot. I No. 1 with Sera of Cancer Patients.

1% solution of Prot. I No. 1 in physiological saline was laid over fresh sera of patients and healthy persons. The results are listed in Table V. Of sera of 12 patients with a malignant tumor, 8 gave the positive reaction but of those of 10 persons, non-cancerous and healthy, only 1 (duodenal ulcer) behaved similarly.

TABLE V.

Precipitin Reaction of Prot. I No. 1 upon Sera of Patients and Healthy Persons.

No. of individuals	Clinical diagnosis	Dilution of sera		
		Undiluted	1:5	1:10
1	Gastric carcinoma	−	−	−
2	„	+	+	−
3	„	+	+	−
4	Cardia carcinoma	+	±	−
5	Rectal carcinoma	−	−	−
6	Esophageal cacinoma	+	±	−
7	Gastric ulcer	−	−	−
8	Retroperitoneal sarcoma	+	+	−
9	Duodenal ulcer	+	−	−
10	Weil's disease	−	−	−
11	Acute liver cirrhosis	−	−	−
12	Gastric ulcer	−	−	−
13	Cholelithiasis	−	−	−
14	„	−	−	−
15	Uterine carcinoma	+	+	−
16	„	+	±	−
17	„	−	−	−
18	„	+	−	−
19	Gastric carcinoma	±	−	−
20	Healthy person	−	−	−
21	„	−	−	−
22	„	−	−	−

Further Properties of Prot.-I's.

With respect to digestibility by pepsin and trypsin, optical rotation, the site of I.I.P., acid-binding force and amino index, Prot.-I's that had been obtained from the carcinomatous ascitic fluids originating in stomach cancer did not definitely differ from the corresponding protein fraction of the other kinds of ascitic fluid.

Digestibility.

Prot.-I's of the ascitic fluids Nos. 1 and 2 were taken for the experiment as representatives of the serologically different groups. As a control experiment caseinogen was digested.

1) Pepsin experiment.

Substrate solutions. The substances were dissolved in a Sörensen's buffer solution of pH 2.0 in such a proportion that 100 cc. of the solutions contained 1 g. of a substance.

Enzyme solution. It was prepared by dissolving 200 mg. Park Davis pepsin U.S.P. 1:3000 and filling up to 4 cc. with the buffer solution above.

To 15 cc. of a substrate solution was added 1 cc. of the enzyme solution after warming each of the solutions to 37°C beforehand. The mixtures were immediately placed in a thermostat at the same temperature and at intervals two 1 cc. portions pipetted out for the formol titration (Sörensen) and gasometry of amino nitrogen (micro Van Slyke).

The results are embodied in Table VI and Fig. 1.

TABLE VI.

Peptic Digestion of Prot. I of Ascitic Fluids* Nos. 1 and 2 and Caseinogen.†

150 mg. each of the Prot.-I's were subjected to the digestion. Increases of acidity are expressed in number of cc. of 0.1 n. NaOH for 9.38 mg. of the proteins and those of amino nitrogen in per cent of the proteins.

Substrate / Time of incub. min.	Prot. I of No. 1 ascitic fluid			Prot. I of No. 2 ascitic fluid			Caseinogen
	Acidity increase	Amino nitrogen		Acidity increase	Amino nitrogen		Acidity increase
		Content	Increase		Content	Increase	
0		1.56			1.40		
2	0.034	1.66	0.10	0.031	1.46	0.06	0.030
5	0.061	1.82	0.26	0.050	1.60	0.20	0.072
10	0.091	1.94	0.38	0.070	1.75	0.35	0.103
20	0.114	2.16	0.60	0.093	1.95	0.55	0.140
30	0.125	2.30	0.74	0.113	2.05	0.65	0.160
60	0.153	2.66	1.10	0.138	2.40	1.00	0.172

* The numbers of the fluids agree with the corresponding numbers in Table I.

† Caseinogen (Hammarsten) E. Merck.

2) Trypsin experiment.

Substrate solutions. They were prepared by using so much Sörensen's buffer solution of pH 8.5 as to contain 1 g. of a substrate per 100 cc.

Enzyme solution. 200 mg. of trypsin G. Grübler were dissolved in 4 cc. of the same buffer solution as above.

For incubation 15 cc. of a substrate solution and 1 cc. of the enzyme solution, both heated to 37°C, were mixed together and kept furtheron at the same temperature, and increases of acidity and amino-nitrogen were

Fig. 1. Peptic digestion. * Prot. I of the ascitic fluid No. 1. † Prot. I of the ascitic fluid No. 2.

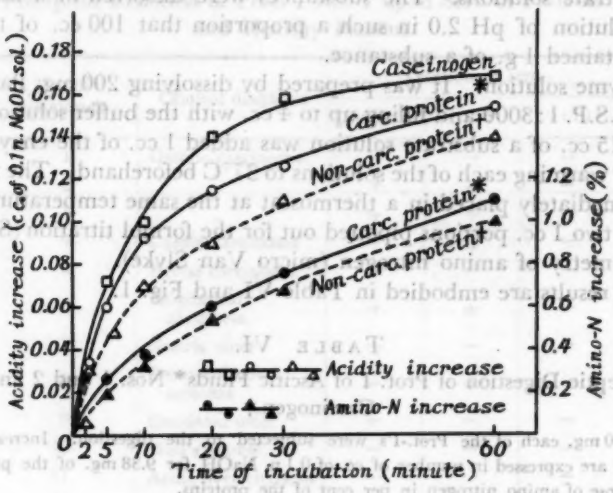


TABLE VII.

Tryptic Digestion of Prot. I of Ascitic Fluids* Nos. 1 and 2 and Caseinogen.†

150 mg. each of the proteins were subjected to digestion. Increases of acidity are expressed in number of cc. of 0.1 n. NaOH for 9.38mg. of the proteins and those of amino nitrogen in per cent of the proteins.

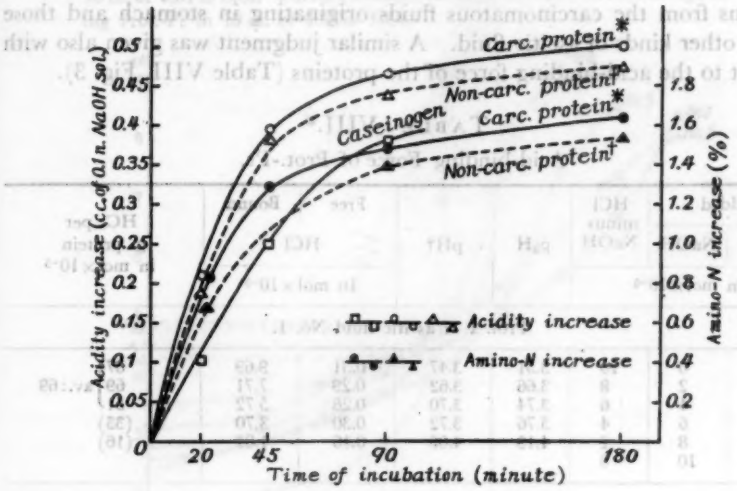
Substrate / Time of incub. min.	Prot. I of No. 1 ascitic fluid			Prot. I of No. 2 ascitic fluid			Caseinogen
	Acidity increase	Amino nitrogen		Acidity increase	Amino nitrogen		Acidity increase
		Content	Increase		Content	Increase	
0		1.53			1.40		
20	0.206	2.38	0.85	0.195	2.14	0.74	0.110
45	0.396	2.80	1.27	0.380	2.56	1.16	0.252
90	0.464	3.01	1.48	0.440	2.80	1.40	0.373
180	0.495	3.16	1.63	0.474	2.94	1.54	0.410

* and † signify the same as in Table VI.

traced as in 1). (Table VII and Fig. 2)

In neither of the two experiments no noticeable divergency of digestibility was found between the two proteins.

Fig. 2. Tryptic digestion. * Prot. I of ascitic fluid No. 1. † Prot. I of the ascitic fluid No. 2.



Optical rotation.

The rotation (in water) was measured on Prot. I of the fluids Nos. 1, 2, 3, 4 and 5.

Source of Prot. I. No.	$[\alpha]_D$
1*	$\frac{-0.70\times 1.9769}{0.0194\times 0.9994\times 1} = -71.4°\ (26°C)$
2‡	$\frac{-0.51\times 1.4482}{0.0099\times 0.9998\times 1} = -74.6°\ (25°C)$
3†	$\frac{-0.69\times 2.0015}{0.0191\times 1.0000\times 1} = -72.3°\ (23°C)$
4‡	$\frac{-0.66\times 2.0058}{0.0176\times 0.9998\times 1} = -75.2°\ (23°C)$
5*	$\frac{-0.55\times 2.0012}{0.0154\times 0.9991\times 1} = -72.1°\ (26°C)$

The cancerous ascitic fluids having the primary seat in stomach are marked with asterisk and that having the primary seat in liver with obelisk and the non-carcinomatous ascitic fluids with double obelisk.

Isoionic point and acid-binding force.

112 mg. of Prot. I from the fluids Nos. 1 and 3–6 were dissolved in 10 cc. of 0.01 n. HCl and titrated with 0.01 n. NaOH potentiometrically (hydrogen electrode). The titration curves intersected the one obtained

on 0.01 n. HCl solution at pH's 4.5, 4.0, 4.9, 4.8 and 4.3 respectively, that is, no definite difference was recognized between the isoionic points of the proteins from the carcinomatous fluids originating in stomach and those of the other kinds of ascitic fluid. A similar judgment was given also with respect to the acid-binding force of the proteins (Table VIII, Fig. 3).

TABLE VIII.*

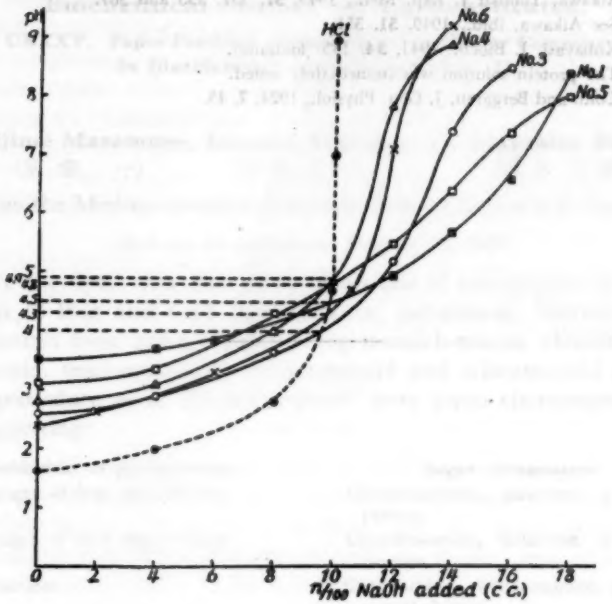
Acid-binding Force of Prot.-I's.

Added HCl	Added NaOH	HCl minus NaOH	p_aH	pH†	Free HCl	Bound HCl	HCl per g. protein in mol×10⁻⁵
In mol×10⁻⁵					In mol×10⁻⁵		
Prot. I of ascitic fluid No. 1.							
10	0	10	3.51	3.47	0.31	9.69	87
„	2	8	3.66	3.62	0.29	7.71	69 av.: 69
„	4	6	3.74	3.70	0.28	5.72	51
„	6	4	3.76	3.72	0.30	3.70	(33)
„	8	2	4.19	4.05	0.16	1.84	(16)
„	10	0					
Prot. I of ascitic fluid No. 3.							
10	0	10	2.58	2.54	2.90	7.10	63
„	2	8	2.80	2.76	2.04	7.96	70 av.: 60
„	4	6	2.97	2.93	1.66	5.34	47
„	6	4	3.29	3.25	0.90	3.10	(27)
„	8	2	3.64	3.60	0.45	1.55	(13)
Prot. I of ascitic fluid No. 4.							
10	0	10	2.74	2.70	2.0	8.0	71
„	2	8	2.90	2.86	1.68	6.32	56 av.: 56
„	4	6	3.01	2.97	1.50	4.50	40
„	6	4	3.47	3.43	0.59	3.41	(30)
„	8	2	4.04	4.00	0.18	1.82	(16)
Prot. I of ascitic fluid No. 5.							
10	0	10	3.12	3.08	0.83	9.17	81
„	2	8	3.26	3.22	0.72	7.28	65 av.: 65
„	4	6	3.42	3.38	0.56	5.44	49
„	6	4	3.82	3.78	0.27	3.73	(33)
„	8	2	4.35	4.31	0.098	1.91	(17)
Prot. I of ascitic fluid No. 6.							
10	0	10	2.42	2.38	4.20	5.80	51
„	2	8	2.62	2.58	3.12	4.88	43 av.: 45
„	4	6	3.01	2.91	1.54	4.46	40
„	6	4	3.37	3.33	0.75	3.25	(29)
„	8	2	3.82	3.78	0.31	1.69	(15)

* Ascitic fluids are numbered as in Table I.

† f_H's of Cohn and Berggren[7] were employed for the calculation.

Fig. 3. Electrometric titration. HCl=the curve obtained by titrating 10 cc. of 0.01 n. HCl. Curves numbered are those obtained on the corresponding Prot.-I's in 10 cc. of 0.01 n. HCl.



Amino index.

Total and amino nitrogen were determined according to Kjeldahl and Van Slyke. Their ratios calculated at ranged from 9.2–11.2 as follows.

Source of Prot. I No.	Total N %	Amino N %	Amino index
1*	15.24	1.56	**10.2**
2‡	15.13	1.40	9.2
3†	15.29	1.61	10.4
4‡	15.34	1.72	11.2
5*	15.43	1.64	**10.6**
6*	15.18	1.67	**11.0**

The marks on the number of the ascitic fluids imply the same as those in the table of optical rotations (p. 233).

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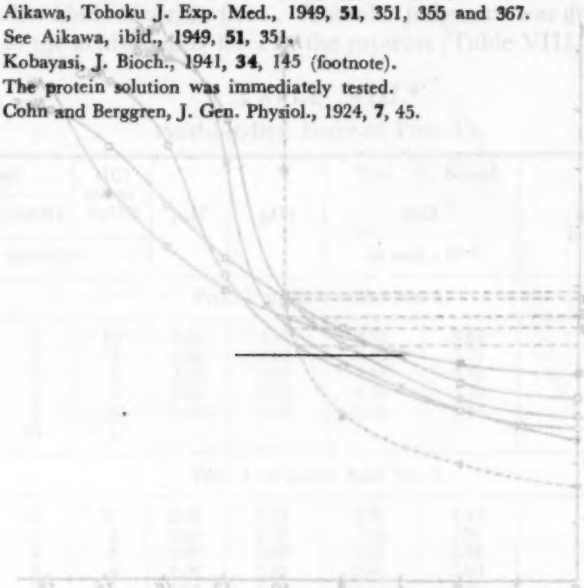
H. Masamune.

References and Notes.

1) Bermbach, Med. Klinik, 1906, No. 12, 307.
2) Sunada and Komiyama, Geka (Surgery), 1948, **10**, 527.
3) Aikawa, Tohoku J. Exp. Med., 1949, **51**, 351, 355 and 367.
4) See Aikawa, ibid., 1949, **51**, 351.
5) Kobayasi, J. Bioch., 1941, **34**, 145 (footnote).
6) The protein solution was immediately tested.
7) Cohn and Berggren, J. Gen. Physiol., 1924, **7**, 45.



The Tohoku Journal of Experimental Medicine, Vol. 53, Nos. 3 & 4, 1951.

Biochemical Studies on Carbohydrates.

CXXXV. Paper Partition Chromatograms of Sugar Components in Glucidamins* and Glycoproteins. II.

By

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Here are dealt with the choromatograms of mucinogens in the egg-coverings of frog and toad, pseudomucin, paramucin, Mörner's water-soluble mucin from urine, mucin of hog stomach-mucus, chondromucoid, osseomucoid, tendomucoid β, corneamucoid and scleramucoid α. By a similar procedure as in the first report[1] were given chomatograms with spots suggesting:

Glucidamin or glycyprotein	Sugar components
Mucinogen of frog egg-covering	Chondrosamine, galactose, a methylpentose
Mucinogen of toad egg-covering	Chondrosamine, galactose, a methylpentose
Pseudomucin	Glucosamine, chondrosamine, galactose, a methylpentose
Paramucin	Glucosamine, chondrosamine, galactose, a methylpentose
Mörner's water-soluble mucin from Group A human urine	Glucosamine, chondrosamine, galactose, a methylpentose
Mörner's water-soluble mucin from Group B human urine	Glucosamine, chondrosamine, galactose, a methylpentose
Mucin of hog stomach-mucus	Glucosamine, chondrosamine, galactose, mannose, a methylpentose
Chondromucoid from whale nasal-cartilage	Glucosamine, chondrosamine, galactose, glycuronic acid
Chondromucoid from bull trachea	Glucosamine, chondrosamine, galactose, glycuronic acid, a methylpentose
Osseomucoid (from bull femur)	Glucosamine, chondrosamine, galactose, glycuronic acid
Tendomucoid β	Glucosamine, chondrosamine, galactose, glycuronic acid
Corneamucoid	Glucosamine, chondrosamine, galactose, glycuronic acid
Scleramucoid α	Glucosamine, chondrosamine, mannose, glycuronic acid

* The term "glycidamin" is more appropriately spelt as glucidamin. H. Masamune.

These findings coincide with those already reported, in so far as the mucinogen in egg-coverings of frog and toad,[2] chondromucoid from nasal cartilage of whale,[3] osseomucoid,[4] tendomucoid β[5] and scleramucoid α[6] are concerned. As to pseudomucin, Turumi and Sasaki[7] noticed the presence of a methylpentose in it as early as 1942, but chondrosamine has been overlooked. Further in former investigations, we have missed methylpentose in paramucin,[8] Mörner's water-soluble mucin from human urine[9] and chondromucoid from bull trachea;[3] chondrosamine, mannose and a methylpentose in the mucus-mucin of hog stomach;[10] and chondrosamine and glycuronic acid in corneamucoid.[11] The spots in the expected positions of methylpentose were all small except when pseudomucin was examined.

Werner and Odin's[12] chromatograms of corneamucoid showed a spot of mannose, though very weak, but their solvent mixture could not resolve chondrosamine and glucosamine.

Experimental.

Materials.

Pseudomucin. By repetition of Hammarsten's preparation procedure[13] on the viscous content of an ovarian cyst was obtained only a substance containing nitrogen not less than 13%. However, electrodialysis of it gave precipitates, which as dried up resembled the preparations of the author regarding N content and solubility. The product contained 10.65% nitrogen, 25.0% hexosamine and 0.03% ash. Non-amino sugars amounted, when expressed as galactose, to 15.2% by the orcinol method, 15.0% by the indole method and 25.3% by the diphenylamine method I. The details will be described elsewhere.[14]

Other materials were previous preparations:

Mucinogens of frog and toad egg-covering by Hiyama,[2]
Paramucin by Hiyama,[8]
Mörner's water-soluble mucin from Group A human urine by Kobayasi and others,[15]
Mörner's water-soluble mucin from Group B human urine by Kobayasi and others,[15]
Mucin of hog stomach-mucus by Sinokawa,[10]
Chondromucoid from nasal cartilage of whale by Hisamura,[3]
Chondromucoid from bull trachea by Hisamura,[3]
Osseomucoid from bull femur by Hisamura,[4]
Corneamucoid by Suzuki,[11]
Tendomucoid β by Sasaki,[5]
Scleramucoid α by Osaki.[6]

Technique of Chromatography of the Sugar Components.

The method was that employed in the study of the blood group carbohydrate from hog stomach-mucus.[16]

Hydrolysis of the materials was effected by heating with 1 n. H_2SO_4 (as sealed in an ampoule) for 5 hours in a boiling water-bath, and after removing H_2SO_4 with baryta, the obtained solutions were condensed to put on the paper. The amounts of materials taken for the hydrolysis and other items will be tabulated:

Material	Amount of materials hydrolyzed	Quantity of 1 n. H_2SO_4 used for hydrolysis	Approximate volume of hydrolysates after condensation	Approximate volume put of condensates
	mg.	cc.	cc.	cc.
Mucinogen of frog egg-covering	50	5	1	0.015
Mucinogen of toad egg-covering	50	5	1.2	0.018

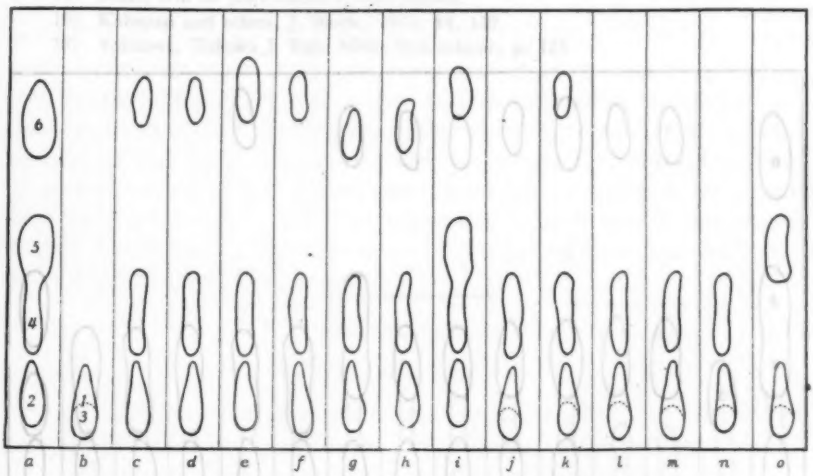

Fig. 1. *n*-Butylacetate-pyridine-water (5:3:1) chromatograms developed with $AgNO_3$-ammonia (duration of test, about 8.5 hrs.; temp., 19–20°C).
a and b: references. c: mucinogen of frog egg-covering. d: mucinogen of toad egg-covering. e: pseudomucin. f: paramucin. g: Mörner's water-soluble mucin from Group A urine. h: Mörner's water-soluble mucin from Group B urine. i: mucin of hog stomach-mucus. j: chondromucoid from whale nasal-cartilage. k: chondromucoid from bull trachea. l: osseomucoid from bull femur. m: tendomucoid β. n: corneamucoid. o: scleramucoid α. 1: chondrosamine. 2: glucosamine. 3: glycuronic acid. 4: galactose. 5: mannose. 6: rhamnose.

Pseudomucin	50	5	1.0	0.02
Paramucin	50	5	0.8	0.02
Mörner's water-soluble mucin fr. Group A urine	100	10	1.2	0.016
Mörner's water-soluble mucin fr. Group B urine	100	10	1.0	0.015
Mucin of hog stomach-mucus	50	5	1.0	0.01
Chondromucoid from whale nasal-cartilage	100	10	1.0	0.025
Chondromucoid from bull trachea	100	10	0.8	0.023
Osseomucoid from bull femur	70	7	0.8	0.025
Tendomucoid β	100	10	1.2	0.025
Corneamucoid	40	4	0.8	0.018
Scleramucoid α	70	7	1.2	0.027

In the control runs 0.015 cc. each of sugar solutions (references) were taken which were 1% with respect to each individual sugar.

The chromatograms are shown in diagrams (Figs. 1 and 2).

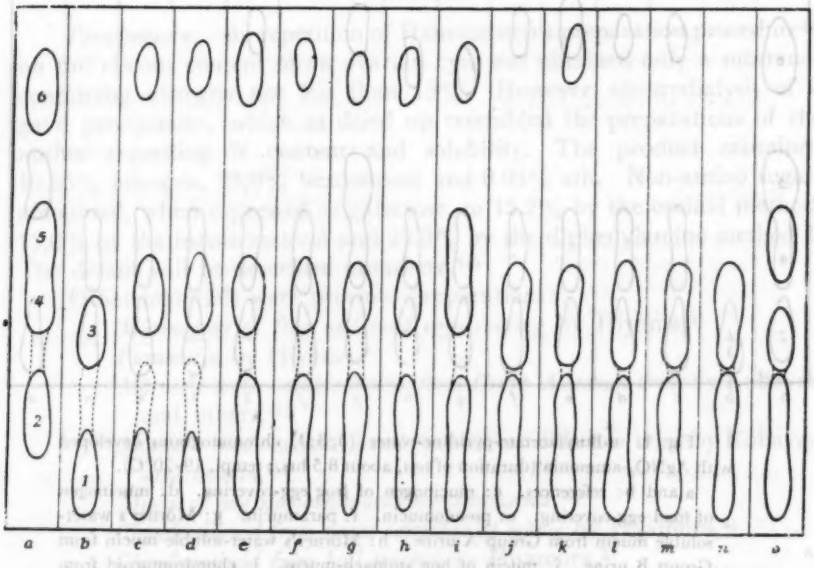

Fig. 2. *n*-Butylacetate-acetic acid-water (3:2:1) chromatograms developed with $AgNO_3$-ammonia (duration of test, about 15.5 hrs.; temp., 19–20°C).

a–o and 1–6 signify the same as corresponding signs and numbers in Fig. 1.

Through the Grant Committee of the Science Council was given from the Education Department a grant in aid to us. H. M.

References.

1) Masamune and Yosizawa, Tohoku J. Exp. Med., this volume, p. 155.
2) Hiyama, ibid., 1949, **50**, 373, 379 and 385.
3) Hisamura, J. Bioch., 1938, **28**, 217; Masamune and Yasuoka, Tohoku J. Exp. Med., 1947, **49**, 181; Hiyama, ibid., 1949, **51**, 65.
4) Hisamura, J. Bioch., 1938, **28**, 473; Hiyama, Tohoku J. Exp. Med., 1949, **51**, 97.
5) Sasaki, Tohoku J. Exp. Med., 1945, **48**, 307.
6) Osaki, ibid., 1947, **49**, 19.
7) Turumi and Sasaki, ibid., 1947, **49**, 1.
8) Hiyama, ibid., 1949, **51**, 327.
9) Kobayasi, J. Bioch., 1939, **30**, 451.
10) Sinokawa, Tohoku J. Exp. Med., 1949, **50**, 167.
11) Suzuki, J. Bioch., 1939, **30**, 185.
12) Werner and Ödin, Upsala Läkarefören. Förhandl. N.F. 1948–49, **54**, 69.
13) Hammarsten, Z. f. physiol. Chem., 1882, **6**, 194.
14) Maki, Will be contributed to this journal.
15) Kobayasi and others, J. Bioch., 1941, **34**, 149.
16) Yosizawa, Tohoku J. Exp. Med., this volume, p. 125.

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Proteolytic Activity of Human Gastric Mucosa.

I.

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In the precise study of the proteolysis of gastric mucosa (swine) Willstätter and Bamann[1] found, besides pepsin (pH 2.0) proper, a proteinase named cathepsin by him which hydrolysed gelatin, clupein and egg albumin at pH 3.5–4.0. In addition to these he confirmed the ereptase activity (pH 8.0, leucyl-diglycin & diglycin splitting), but not tryptase activity, because the hydrolysis of gelatin at pH 8.0 was negative (48 hrs.).

Recently Hirooka[2] has in his studies with swine gastric mucosa, successfully separated the pepsin activity from the erepsin and cathepsin activity on one hand, and also the catheptic pepton splitting activity from erepsin by means of acid and alkaline treatment according to the method of Utzino and Sakai.[3] It will be interesting to study exactly the protease activity of the human gastric mucosa, by comparing with such results from experiments with animal materials.

Experimental.

Method and Materials.

1) Enzyme: Macroscopic normal gastric mucosa from cancer- and ulcer-stomach obtained by biopsy. Maceration 1+glycerin-water (1:1)3.

Acid and alkaline treatment: according to Utzino and Sakai.[3]

Cysteine activation: 2 c.c. enzyme+0.024 grm. cystein-HCl in 1 c.c. H_2O (neutralized with NaOH), 37°C, 30 min., for control when needed, the volume of the original enzyme solution was made constant, with buffer solution.

2) Substrate: 1–2 % for m/20-substrate-buffer-solution; acylpeptid or acylamino acid resolved at first in n/10 NaOH then added with buffer; buffer: m/10-citrate-n/10-HC or m/15-phosphate-buffer according to the conditions of experiments.

Reactions: 18–20 c.c. Substrate-buffer solution+2–3 c.c. enzyme

solution, under toluol, at 37°C; for control: without substrate or enzyme under the same condition; increased acidity in 4 c.c. digestate formol-titrated (0.1 n-NaOH). Only corrected values are given in the tables.

Results and Discussion.

I. Hydrolysis of protein and pepton.

Besides pepsin action (pH 2.0), hydrolyses of casein, serum albumin and egg albumin and also pepton between pH 4.0 and 5.0 are recognized (cathepsin action, Table I), which are with the addition of cystein apparently activated (pH 4.0 Table II). Further it must be attended that casein also at pH 8.5 is hydrolysed, while pepton without strangeness is (erepsin activity) (Table I).

TABLE I.

Proteolysis of Human Gastric Mucosa.

2 c.c. maceration juice; enzyme no. 173 & 179: cancer.

Increased acidity in 4 c.c. digestate, c.c. 0.1 n-NaOH

Substrate 18 c.c.	Time in hrs.	pH								
		2.0	3.0	4.0	4.4	4.6	5.0	6.0	7.5	8.5
1% Casein (Hammarsten)	24	0.4	0.4	0.5	0.25	0.25	0.2	—	0.1	0.15
	48	0.5	0.4	0.55	0.35	0.35	0.3	—	0.2	0.2
	72	0.55	0.4	0.6	0.4	0.4	0.4	—	0.35	0.45
1% Pepton (Kyokuto)	24	−0.05	—	0.35	—	0.2	—	0.3	0.6	0.3
	48	0.05	—	0.45	—	0.3	—	0.5	0.6	0.45
	72	0.1	—	0.5	—	0.55	—	0.6	0.9	0.55

After the acid treatment (pH 4.0, 40 min. 37°C) for the maceration disappears the erepsin activity, while pepton hydrolysing activity at pH 4.0 is reserved (Table III). And after the alkaline treatment (pH 7.7, 30 min., 37°C), the degrees of hydrolysis of casein, egg albumin and serum albumin at pH 4.0 become smaller than that of these substrates at pH 2.0–2.2, without being activated with cystein (Table V). On the contrary, the degrees of hydrolysis of these substrates at pH 4.0 by original maceration juice are simillar to those at pH 2.0, and the former can be activated with cystein as has been seen in Table II (Table V), though the latter might not be. From these it is evident that the cathepsin action was removed by alkaline treatment. However, the pepton hydrolysing activity at pH 4.0 is reserved even after a stronger treatment with alkaline (pH 9.5,2 hrs., 37°C (Table IV). This fact indicates that the hydrolyzing activity of casein and that of pepton at pH 4.0 differ from each other in

TABLE II.

Catheptase Activity of Human Gastric Mucosa.

2 c.c. maceration juice; enzyme no. 179: cancer; pH 4.0.

Substrate 18 c.c.	Time in hrs.	Increased acidity in 4 c.c. digestate, c.c. 0.1 n-NaOH: With cystein	Without cystein
0.5 % Ovalbumin (Takeda)	24	0.2	0.1
	72	0.3	0.1
	120	0.3	0.15
1 % Ovalbumin (E. Merck)	24	0.4	0.3
	72	0.5	0.3
	120	0.6	0.4
1 % Serum albumin (E. Merck)	24	0.5	0.3
	72	0.5	0.4
	120	0.7	0.55
1 % Casein (Hammarsten)	24	0.65	0.4
	72	0.8	0.6
	120	0.9	0.65
1 % Pepton (Kyokuto)	24	0.4	0.1
	72	0.5	0.3
	120	0.65	0.35

TABLE III.

Catheptic Pepton Splitting Activity of Human Gastric Mucosa (free from ereptase activity).

3 c.c. maceration; enzyme: no. 173, cancer.

Substrate 18 c.c.	Time in hrs.	Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH: pH 3.0	4.0	5.4	7.4	8.4
1 % Pepton (Kyokuto)	24	0.1	0.3	0.1	0	0
	48	0.15	0.4	0.25	0	0

TABLE IV.

Pepton Splitting Activity After the Alkaline Treatment.

3 c.c. maceration; enzyme: no. 173, cancer.

Substrate 18 c.c.	Time in hrs.	Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH: pH 3.0	4.0	5.4	6.8	7.8
1 % Pepton (Kyokuto)	24	0.05	0.15	0.05	0.1	0.2
	48	0.1	0.2	0.1	0.15	0.3
	72	0.1	0.3	0.15	0.15	0.35

relation to their resistency against alkaline. From these respects it may be concluded that in human gastric mucosa does exist catheptic pepton splitting enzyme (cathopeptonase) which was found by Sakai[4] for the first time in swine liver.

Furthermore, tryptase activity in gastric mucosa, which has been denied by Willstätter,[1] probably exists in gastric mucosa, for the hydrolysis of casein at pH 8.5 is positive, which does not seem resulting from reversed and infiltrated pancreas trypsin, for the gastric mucosa employed was obtained from the cancer stomach affected with pyloric stenosis.

II. Hydrolyses of peptide and its derivates.

The hydrolyses of diglycin and chloracetyl-l-leucin at pH 7.8 are clearly positive but that of chloroacetyl-l-phenylalanin is still smaller than those of the former (Table VI). Chloracetyl-diglycin is hardly hydrolyzed by mucosa from cancer stomach, but a little by that from ulcer stomach

TABLE V.

The Pepsinase Activity of Human Gastric Mucosa (free from the cathepsin activity).

2 c.c. maceration; enzyme: no. 173, cancer, alkaline treatment A (+), B (—).

Substrate 18 c.c.	Enzyme	Time in hrs.	Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH					
			With cystein				Without cystein	
			pH				pH	
			2.2	3.0	4.0	5.0	0.4	2.0
1% Casein (Hammarsten)	A	24	0.3	0.1	0.1	0.1	0.1	0.35
		48	0.4	0.2	0.1	0.15	0.15	0.4
		72	0.5	0.25	0.2	0.15	0.15	0.5
	B	24	0.4	—	0.65	—	—	0.4
		48	0.4	—	0.8	—	—	0.45
		72	0.6	—	0.8	—	—	0.5
1% Ovalbumin (E. Merck)	A	24	0.3	—	0.2	—	0.2	0.3
		48	0.3	—	0.2	—	0.2	0.3
		72	0.6	—	0.3	—	0.25	0.4
	B	24	0.25	—	0.4	—	0.3	—
		48	0.3	—	0.4	—	0.3	—
		72	0.4	—	0.6	—	0.4	—
1% Serum albumin (E. Merck)	A	24	0.45	—	0.25	—	0.2	—
		48	0.55	—	0.3	—	0.2	—
		72	0.6	—	—	—	0.3	—
	B	24	0.45	—	0.6	—	0.3	—
		48	0.55	—	0.6	—	0.45	—
		72	0.6	—	0.7	—	0.6	—

(Table VI). The extent of hydrolysis of these halogen-acyl-amino-acids by human gastric mucosa is nearly equal to that by intestinal juice (dog and also to that by intestinal mucosa (rabbit), which were observed in the experiments of Matsuo[5] and Hino[6] respectively. And it is of interest that it can be observed clearly even when the hydrolysis of casein at pH 8.5 is still yet questionable (24 hrs.) (Tables I & VI). From these results the existence of halogen-acylase activity in human gastric mucosa may be concluded. On the contrary the hydrolyses of benzoyl-diglycin and benzoyl-glycin were by no means recognized (Table VI).

Then influence of the kinase[7] activation was examined: hydrolysis of diglycin is not affected, that of benzoyl-diglycin is still questionable, but

TABLE VI.

Peptide Splitting Activity of Human Gastric Mucosa (1)
Experiments in Alkaline Side.

2 c.c. maceration; pH 7.8.

Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH

m/20 Substrate 18 c.c.	Time in hrs.	Enzyme, no.				
		196 cancer	194 cancer	177a cancer	207 ulcer	177b ulcer
Diglycin	24	1.60	1.70	1.50	1.60	1.40
	48	1.60	1.85	1.50	1.60	1.60
	72	1.70	1.95	1.85	1.65	1.60
Benzoyldiglycin	24	0	0.10	0	0	0
	72	0	0.10	0	0	0
	120	0.15	0.10	0.15	0.05	0
Chloracetyl-l-phenyl-alanin	24	0.30	—	—	0.50	—
	72	0.45	—	—	0.70	—
	120	0.60	—	—	0.80	—
Chloracetyl-l-leucin	24	0.60	0.45	0.65	1.00	0.95
	72	0.75	—	0.80	1.85	1.40
	120	1.20	—	0.85	2.10	1.70
Chloracetyldiglycin	24	0	—	0	0.25	0.25
	72	0.20	—	0	0.30	0.30
	120	0.20	—	0.25	0.40	0.35
Bezoylglycin	24	−0.05	—	0	0	0
	72	−0.10	—	0	0	0
	120	−0.15	—	0.10	0	−0.05

that of chlor-acetyl-diglycin seems to have undergone a little activation (Table VIII).

By the by, halogen-acylase activity of cancer stomach is weaker than that of ulcer stomach, while no difference with the dipeptidase activity is found between these two.

In the experiments in acid side, neither acid carboxypeptidase activity (benzoyl-diglycin, Waldschmidt-Leitz[8]) nor acid acyl-dipeptidase activity (benzoyl-diglycin, Sakai[9]) is found in human gastric mucosa. This is also the case still with cystein activation (Table VII).

SUMMARY.

1. In human gastric mucosa besides peptic action, are found cathepsin action (pH 4.0–5.0, casein, egg albumin and serum albumin) and catheptic

TABLE VII.

Peptide Splitting Activity of Human Gastric Mucosa (2)
Experiments in Acid Side.

3 c.c. maceration juice; cystein activation A (+), B (—); pH 4.2–4.6.

Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH

m/20 Substrate 18 c.c.	Time in hrs.	Enzyme, no, (pH)						
		no. 225 ulcer (4.4)		no. 233 ulcer (4.2)	no. 209 cancer (4.6)		no. 196 cancer (4.5)	no. 194 cancer (4.5)
		A	B	B	A	B	A	A
Diglycin	24	0.10	0	0	—	—	−0.05	0
	72	0.10	0	0	—	—	−0.10	0
	120	—	—	—	—	—	−0.10	0
Chloracetyl-l-phenylalanin	24	0.05	—	—	0.05	0	0	—
	72	0.10	—	—	0.05	0.05	−0.05	—
	120	—	—	—	−0.10	0.05	−0.10	—
Chloracetyldiglycin	24	0.05	0	—	—	0.05	0	—
	72	0.05	0	—	—	0.05	0.05	—
	120	—	—	—	—	0.05	−0.05	—
Chloracetyl-l-leucin	24	0	0	0	0	0	−0.05	—
	72	0.05	0	0	0	0	−0.05	—
	120	—	—	—	−0.05	0.05	−0.10	—
Benzoyldiglycin	24	0.05	0	—	0.05	0.10	0	0
	72	0.05	0	—	0.05	0.10	0	0
	120	—	—	—	−0.10	0.20	−0.10	−0.05
Benzoylglycin	24	0.05	0	—	—	—	0	—
	72	0.05	0.05	—	—	—	0	—
	120	—	—	—	—	—	−0.05	—

pepton splitting action (cathopeptonase). And casein is also at pH 8.5 hydrolized (72 hrs.).

2. I have succeeded also with human gastric mucosa to separate the pepsin action from the cathepsin action, and also the cathopeptonase action from the erepsin action by means of acid and alkaline treatment respectively according to the methods of Utzino and Sakai.[3]

3. In human gastric mucosa halogen-acylase action (pH 7.8, chloracetyl-l-leucin and chloracetyl-l-phenylalanin) is found, but carboxypeptidase action (pH 7.8, benzoyl-diglycin, with kinase) or hippuricase action (pH 7.8, benzoyl-glycin) not. The hydrolysis of chlor-acetyl-

TABLE VIII.

Peptide Splitting with Kinase Activation.

2 c.c. maceration+0.05 grm. kinase pulver, 37°C 30 min.; enzyme: No. 204, ulcer; m/20-substrate; Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH; pH 7.8.

Substrate 18 c.c.	Time in hrs.	Without activation	With activation
Diglycin	24	1.30	1.50
	48	1.40	1.75
	72	1.45	—
Benzoylglycin	24	0	0.20
	48	0	0.20
	72	−0.05	—
Chloracetyldiglycin	24	0.10	0.50
	48	0.30	0.60
	72	0.40	—

diglycin at pH 7.8 by gastric mucosa from ulcer stomach is certain extent recognized.

4. Neither acid carboxy-peptidase action nor acid acyldipeptidase action (pH 4.2–4.6, benzoyl-diglycin, chloracetyl-diglycin) despite of the cystein activation is still yet found. The hydrolysis of diglycin, chloracetyl-l-leucin, chloracetyl-phenylalnin and benzoyl-glycin at pH 4.5 with cystein, all of these are negative.

References.

1) Willstätter and Bamann, Ztschr. physiol. Chem., 1929, **180**, 127.
2) Hirooka, Tohoku Journ. Exp. Med., 1942, **42**, 178.
3) Utzino and Sakai, Journ. Biochem., 1941, **33**, 457.
4) Sakai, Journ. Biochem., 1944, **36**, 443.
5) Matsuo, Journ. Biochem., 1941, **33**, 59.
6) Hino, Tohoku Journ. Exp. Med., 1944, **47**, 344.
7) Kawaharada, Journ. Biochem., 1944, **36**, 493.
8) Waldschmidt-Leitz, Schäffner, a.o., Ztschr. physiol. Chem, 1930, **188**, 17.
9) Sakai, Journ. Biochem., 1944, **36**, 383.

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Proteolytic Activity of Human Gastric Mucosa.

II. Its Tryptase Activity.

By

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Tryptic enzyme in animal tissue and serum or in lymphocyte named "tissue trypsin" has been described since early, which should be dealt with as an enzyme "tryptase" in general, discriminated from pancreas trypsin, while it is yet questionable whether the former is identical to the latter or not. Although its activity is so weak that it is impossible to observe it as separated from that of cathepsin, the apparent hydrolysis of casein and gelatin at pH 7.8 by intestinal mucosa in the Thiry-Vella's fistula (dog), where an action of pancreas trypsin is excluded, were demonstrated by Matsuo[1] recently (72 hrs.). These hydrolyses were attributed to an action of "enterotryptase" and activated in the presence of the dialyzed albumin to a high extent. It is also the case with the experiments by Hino[2] who studied further with intestinal mucosa of a rabbit and with egg albumin.

In the previous paper I[3] pointed out that casein was at pH 8.5 also by human gastric mucosa hydrolysed. In view of these respects I intend to study further about the tryptase activity of human gastric mucosa.

Experimental.

Method and Materials.

Dialysed albumin solution: 2–4% egg albumin (E. Merck) in m/15-phosphate-buffer solution (pH 7.8) was dialyzed in cellophane sack for 7–10 days against service water and then for 1–2 days against aq. dest.

Materials were obtained from biopsy.

Results and Discussion.

The hydrolysis of casein, which was already seen in the previous paper, is apparently after the long digestion of 72 hrs., having its optimal pH range between 7.2 and 8.4, but that of gelatin remains still questionable

and those of serum albumin and of egg albumin are negative (Table I). After the maceration juice was centrifugated, the precipitate suspension is still capable of hydrolysing casein, pepton and diglycin, but not chloracetyl-l-leucin. On the contrary, the supernatant can hydrolyse all of these substrates (Table II). At that time it is of interest to think the fact that the degree of the hydrolysis of chloracetyl-l-leucin by the supernatant is nearly equal to that by original enzyme solution together with the above results, for chlor-acetyl-l-leucin is employed by other scholars also as a substrate for carboxypeptidase. And the results show further that part of tryptase in gastric mucosa may also be in desmo-form.

TABLE I.

Tryptase Activity of Human Gastric Mucosa.

Enzyme no. 200, ulcer; 2 c.c. maceration juice.

Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH

% Substrate 18 c.c.	Time in hrs.	pH				
		7.2	7.6	7.8	8.0	8.4
1% Casein (Hammarsten)	24	0.15	0.15	0.15	0.3	0.25
	74	0.2	0.4	0.5	0.45	0.3
	120	0.3	0.5	0.65	0.7	0.4
1% Gelatin (Kokko)	24	—	—	−0.05	0	0
	72	—	—	0.1	0.1	0
	120	—	—	0.1	0.2	—
2% Serum albumin (E. Merck)	24	—	—	—	−0.05	—
	72	—	—	—	−0.1	—
	120	—	—	—	−0.15	—
2% Ovalbumin (E. Merck)	24	—	—	—	−0.05	—
	72	—	—	—	0.1	—
	120	—	—	—	0.1	—
2% Ovalbumin (Takeda)	24	—	—	0	0	—
	72	—	—	0	0	—
	120	—	—	0	−0.05	—

To examine whether such a hydrolysis of casein as mentioned above resulted from reversed pancreas trypsin or not, the comparison was made of hydrolysis by enough washed mucosa and by simply washed one: no difference was found between each other (Table III). This proves that examination resulted in the negative. Moreover, as is obvious from the results of the experiment with cystein, casein hydrolysis is by no means a result from the cathepsin action, because it is rather inhibited by cystein (Table IV). Kinase[4] activation with which hydrolyses of egg albumin and serum albumin are still negative, shows no effect upon hydrolysis of

Table II.

Ereptase and Tryptase Activity of Human Gastric Mucosa.

2 c.c. Maceration juice was centrifugated. The precipitate was resolved in buffer solution and made its volume equal to the original maceration juice. pH 7.8; Substrate: 20 c.c.

Substrate	Time in hrs.	Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH: Original enzyme	Supernatant	Precipitate
1% Casein (Hammarsten)	24	0.15	0.15	0.1
	72	0.45	0.3	0.2
	120	0.55	0.5	0.3
1% Pepton (Kyokuto)	24	0.3	0.4	0.1
	72	0.35	—	0.25
	120	0.5	—	—
m/20 Diglycin	24	1.2	0.95	0.9
	72	1.35	1.1	1.05
	120	1.75	1.2	1.1
m/20 Chlor-acetyl-l-leucin	24	0.4	0.35	0
	72	—	0.4	0.05
	120	—	0.5	0.05

Table III.

Tryptase Activity before and after the Washing with Water.

2 c.c. Maceration juice; enzyme no. 201, cancer and 202, ulcer; pH 7.8; Substrate: 20 c.c. casein (Hammarsten); Increased acidity in 4 c.c. digestate: c.c. 0.1' n-NaOH.

Time in hrs.	Simply washed	Enough washed
24	0.1; 0.15	0.2 ; 0.15
72	0.2; 0.252	0.45; 0.3
120	0.7; 0.65	0.7 ; —

Table IV.

Tryptase Activity with Cystein Activation.

2 c.c. Maceration juice+0.024 grm. Cystein-HCl, 37°C, 30 min.; enzyme no. 204, cancer; substrate: 20 c.c. 1% casein (Hammarsten); pH 7.8; increased acidity in 4 c.c. digestate: c.c. 0.1 n-NaOH.

Time in hrs.	Without cystein	With cystein
24	0.15	0.05
72	0.45	0.15
120	0.75	0.25

casein or gelatin (Table V). Further each hydrolysis of casein, gelatin, pepton and diglycin remains without any effect also in the presence of egg albumin or serum albumin (Tables VI & VII). Moreover, even when the enzyme is so diluted its tryptic activity is easily be affected, the hydrolysis of casein is not inhibited by human serum, while that of gelatin seems to undergo little retardation (Table VIII). As the results of the control experiments in Tables VI & VII reveal, serum albumin and egg albumin

Table V.

Tryptase Activity with Kinase Activation.

2 c.c. Maceration juice (enzyme no. 204, cancer) + 0,05 grm. kinase pulver, 30 min. 37°C; pH 7.8.

		Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH	
% Substrate 18 c.c.	Time in hrs.	Without activation	With activation
1% Casein (Hammarsten)	24	0.2	0.2
	72	0.5	0.65
	120	0.9	0.9
1% Gelatin (Kokko)	24	0.1	0.1
	72	0.25	0.3
	120	0.5	0.55
2% Serum albumin (E. Merck)	24	0	0
	72	0.1	0.1
	120	0.25	0.2
2% Ovalbumin (Takeda)	24	0	0
	72	0	0
	120	0.05	−0.05

Table VI.

Tryptase Activity of Human Gastric Mucosa in the Presence of Ovalbumin.

2 c.c. Maceration juice; enzyme no. 222, cancer; pH 7.8.

		Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH.				
Substrate 10 c.c.	Time in hrs.	Without albumin	With 10 c.c. albumin solution (%, concentration)			
			0.5	1	2	4
2% Casein (Hammarsten)	24	0.2	0.15	0.15	0.1	0.2
	72	0.5	0.5	0.45	0.4	0.3
	120	0.65	—	—	0.7	0.7
2% Gelatin (Kokko)	24	0.1	—	—	—	0.1
	72	0.2	—	—	—	1.2
	120	0.25	—	—	—	—
2% Pepton (Kyokuto)	24	0.3	0.25	0.25	0.3	0.3
	72	0.4	0.25	0.3	0.35	0.35
	120	0.5	—	0.5	0.45	0.5
m/20 Diglycin	24	0.75	—	—	—	0.7
	72	0.8	—	—	—	0.95
	120	0.9	—	—	—	1.05
Control without substrate	24	0	0	0	0	0
	72	0.05	0.05	0	0.05	0.05
	120	0.05	0.05	0.1	0.1	0.1

Corrected values are given in the table.

Table VII.

Tryptase Activity in the Presence of Serum Albumin.

Serum albumin; E. Merck; enzyme no. 222, cancer; 2 c.c. maceration juice+10 c.c. substrate solution+10 c.c. albumin solution or buffer; pH 7.8.

Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH						
Substrate 10 c.c.	Time in hrs.	Without albumin	With 10 c.c. albumin solution (%, conc.)			
			0.5	1	2	4
2% Casein (Hammarsten)	24	0.15	0.15	0.15	0.15	0.15
	72	0.4	0.45	0.4	0.35	0.4
	120	0.55	—	0.45	0.45	0.45
2% Gelatin (Kokko)	24	0.1	—	—	—	0.1
	72	0.25	—	—	—	0.2
	120	0.3	—	—	—	0.3
2% Pepton (Kyokuto)	24	0.3	0.25	0.2	0.3	0.3
	72	0.4	0.35	0.35	0.4	0.4
	120	0.55	—	0.4	0.45	0.5
m/20 Diglycin	24	0.9	—	—	—	0.9
	72	0.95	—	—	—	0.95
	120	1.05	—	—	—	1.1
Control without substrate	24	0	0	−0.05	0	0.05
	72	0	0	0	0.05	0.05
	120	0.05	0.1	0.1	0.05	0.1

Corrected values are given in the table.

are not hydrolyzed in each case. On the other hand the tryptase activity of human gastric mucosa also is activated to a high extent in the presence of dialyzed albumin (Tables IX & X), which coincides with the results observed by Matsuo[1] and Hino[2] who studied with intestinal materials of animals. That is, the apparent increase in the degrees of hydrolyses of casein and gelatin, and cleaving of serum albumin and egg albumin which can usually be hardly recognized as in Tables I & VI etc.

Whether this tryptase should be distinguished as "gastrotryptase" from the "enterotryptase" (Matsuo[1]) or these two enzymes are identical remains unsolved. Provided these are identical and should be named "gastro-entero-tryptase" widely distributed in human and animal intestinal mucosa, it must be distinguished further from tryptase of lymphocyte, as has been recently pointed out by M. Bergmann.[5] In this respect tryptase activity of lymph gland (pH 7.0–8.0, casein and gelatin etc.) observed by Nakamura[6] more recently should be attended to.

By the by, the nature of the activation with dialyzed albumin is still unknown.

Table VIII.

Tryptase Activity of Human Gastric Mucosa in the Presence of Human Serum.

Enzyme no. 228, cancer; 1 c.c. maceration juice+20 c.c. 1% substrate-buffer-solution+a c.c. serum or buffer; pH 7.8.

1% Substrate 20 c.c.	Time in hrs.	Without albumin	Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH. c.c., Serum added		
			0.5	1	2
Gelatin (Kokko)	24	0.05	0.05	0.05	0
	72	0.1	0.05	0.05	0
	120	0.2	—	0.1	0.1
Casein (Hammarsten)	24	0.1	0.1	0.1	0.1
	72	0.3	0.25	0.25	0.3
	120	0.4	0.4	0.45	0.5
Ovalbumin (Takeda)	24	0.05	—	—	0.05
	72	0.05	—	—	0.05
	120	0.05	—	—	0.1
Serum albumin (E. Merck)	24	−0.05	—	—	0
	72	−0.05	—	—	0.05
	120	−0.1	—	—	0.05
Control without substrate	24	0	0	0	0
	72	0	0.05	0.05	0.05
	120	0	0.05	0.05	0.1

Corrected values are given.

Table IX.

Tryptase Activity of Human Gastric Mucosa in the Presence of Dialyzed Ovalbumin.

2 c.c. maceration juice+10 c.c. 2% casein (Hammarsten)-buffer-solution+5 c.c. dialyzed ovalbumin solution or buffer+5 c.c. buffer; pH 7.8; corrected values are given.

Enzyme no.	Time in hrs.	Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH Without albumin	With 5 c.c. albumin	Control without substrate Without albm.	With albm.
213 (cancer)	24	0.3	0.7	0	0.05
	72	0.5	0.79	0	0.05
	120	0.65	1.0	−0.05	0.05
214 (cancer)	24	0.15	0.5	0	0.1
	72	0.4	0.85	0	0.1
	120	0.8	1.05	0	0.1
219 (ulcer)	24	0.2	0.55	0	0.05
	72	0.4	0.7	0.05	0.1
	120	0.6	1.05	0.05	0.2

TABLE X.

Tryptase Activity of Human Gastric Mucosa in the Presence of Dialyzed Ovalbumin.

Enzyme no. 228, cancer: 2 c.c. maceration juice + 10 c.c. 2% substrate-buffer-solution + 10 c.c. serum albumin solution or buffer; pH 7.8.

Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH

Substrate	Time in hrs.	Without albumin	With 10 c.c. 4% ovalbm. not dialysed	With 10 c.c. dialysed ovalbumin
Ovalbumin (E. Merck)	24	0	0	0.3
	72	−0.1	0	0.5
	120	−0.1	0	0.6
Serum albumin (E. Merck)	24	0.05	0	0.2
	72	−0.05	0.05	0.25
	120	−0.05	0.05	0.35
Casein (Hammarsten)	24	0.15	0.15	0.5
	72	0.4	0.4	0.8
	120	0.8	0.8	1.25
Gelatin (Kokko)	24	0.15	0.15	0.5
	72	0.2	0.25	0.75
	120	0.3	0.3	0.9
Control without substrate	24	0	0.05	0.15
	72	0	0.05	0.2
	120	0.05	0.05	0.3

SUMMARY.

1. In human gastric mucosa tryptase activity (pH 7.2–8.4, casein & gelatin) is surely present, but against it serum albumin and egg albumin are usually resistent.

2. Tryptase of human gastric mucosa may partially find itself in desmo-form and its activity remains also in the presence of not dialyzed serum albumin or egg albumin unaffected. The hydrolysis of gelatin seems to undergo some retardation in the presence of human serum but that of casein surely not. The tryptase activity is rather inhibited by cystein, but no effect of kinase activation is found.

3. Tryptase activity of human gastric mucosa undergoes activation in the presence of dialyzed albumin, with which such a protein as egg albumin or serum albumin comes to be hydrolyzed, though it is usually resistent to the tryptase activity.

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References.

1) Matsuo, Tohoku Journ. Exp. Med., 1941, **41**, 108; Journ. Biochem., 1941, **33**, 97.
2) Hino, Tohoku Journ. Exp. Med., 1944, **47**, 344.
3) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 243.
4) Kawaharada, Journ. Biochem., 1944, **36**, 493.
5) Bergmann and Fraenkel, Journ. Biol. Chem., 1946, **166**, 721.
6) Nakamura, Journ. Jap. Biochem. Soc., 1948, **20**, 231.

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Studies on Serum Antitrypsin.

First Report.

Inhibition Mechanism of Serum against Tryptic Proteolysis.

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The expression by Michaelis & Menten[1] or that by Haldane[2] have hitherto been the favorites with many scholars who have made chemical kinetic studies of inhibition mechanism of enzyme action. Those expressions, however, are limited in their application only to certain special cases with many defects about themselves.

Recently a universally applicable theory was demonstrated by Goldtein,[3] the above expressions being therein comprised. Maruo,[4] who also attained to the same equation of the first order inhibition as Goldstein's, further showed that it also could, under certain conditions, be introduced from the theory of adsorption where the reactions take place on the heterogeneous system. In this paper I intend to treat the inhibition mechanism of human blood serum under the substrate-trypsin-serum-system, chiefly following Goldstein's theory.

The Theory of Inhibition Mechanism.

On the assumption that a reversible enzyme inhibitor combination be brought forth, the relation between the enzyme (E) and the inhibitor (I) in the state of equilibrium may through the law of mass action, be expressed as follows;

$$K_I = \frac{(E_f)\,(I_f)}{(EI)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

here (E_f), (I_f) and (EI) denoting molar concentrations of free enzyme, of free inhibitor and of the compound of the both respectively, and K_I the dissociation constant.

As the reaction velocity at its beginning is proportional to the enzyme concentration, the inhibition coefficient i which is obtained from the retardation of the reaction velocity must be given in this expression:

$$i=1-\frac{(E_f)}{(E)}=\frac{(I_f)}{(I_f)-K_I} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

This is the same as Maruo[4] introduced. Now from expressions (1) and (2), we can get the expression (3) by eliminating (E_f) and (I_f), $(EI=E-E_f=I-I_f,\ E_f=\frac{K_I}{I_f-K_I}E)$:

$$I=K_1\frac{i}{1-i}+iE \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Then with the quantitative relation between E and i taken into account, we are to have the following three zones of enzyme behavior[3] (Δi means the minimal value to be measured of the varying i).

i) Zone A, where all inhibitors are in a free state.

$$E<0{,}1\ \Delta i,\ E'=\frac{E}{K_I} \ldots\ldots I=K_I\frac{i}{1-i} \ldots\ldots\ldots \quad (4)$$

ii) Zone B, where expression (3) is generally applied.

$0.1\ \Delta i<E'<100\ \Delta i$

iii) Zone C, where all inhibitors are in combined state.

$$E'>100\ \Delta i \ldots\ldots\ldots I=IE \ldots\ldots\ldots\ldots \quad (5)$$

These theoretical equations hold good only when the amount of hydrolysis of the substrate is proportional to the concentration of the enzyme. Therefore, if the inhibition coefficient i′, which has been obtained from the amount of hydrolysis of the substrate not in proportion to the concentration of the enzyme, should be applied, the three equations (3), (4) and (5) could not be directly satisfied. Accordingly, as is treated below in detail, a new equation must be found with regard to the value of i′ by further studies of how it changes when in the trypsin-substrate-serum-system the quantities of any two of the three are kept constant and only that of the other is varied.

Experimental.

Method.

Enzyme: Pancreatin (Iwaki); Substrate: casein (Hammarsten), both resolved in a m/15-phosphate-buffer solution (pH 8.0 as the optimal pH-value for tryptic proteolysis by pancreatin); Serum: diluted to a needed extent with saline buffer solution.

Incubation was conducted at 38°C under toluol and the acidity increased in 2 c.c. digestate (0.01 n-NaOH) was found by formol titration, which Northrop[5] had adopted. With a view to controlling I tried further experiments in incubation under the same conditions, in which at one

time trypsin was used with or without serum and at another only serum was used. In these cases no substrate was added. The figures given in the table show the corrected values (average values from 3 experiments).

Results.

I. Cases where the concentration of the enzyme solution is varied.

a) When the concentration of the enzyme solution is made relatively low and so reaction conditions seem to be in zone A (provided $\Delta i'=0.05$, then $E'<0.5\ \Delta i'$):

In this case the amount of the hydrolysis by trypsin of the substrate x_0 is not proportional directly to the enzyme concentration but to the square root of it, if the serum is not present. This proves that Schutz's rule is applicable to the relation between these two quantities.

TABLE I-a.

Experiment in which the Enzyme is Varied while the Plasma Kept Constant.

(1) Concentration of the Enzyme Relatively Low.

2.0 c.c. 2% casein+2.0 c.c. enzyme+0.5 c.c. plasma (1:40) 1.5 c.c. buffer or 2.0 c.c. buffer; 24 hrs.

$E\times10^4$ Relat. conc. of enz. %	$\sqrt{E}$	Acidity increased; c.c. 0.01 n-NaOH*		E_f**	Inhibition*** coefficient	
		x_0	x		i	i'
0.04	20	3.77	3.02	14	0.965	0.200
0.02	14	2.83	1.68	8	0.960	0.410
0.01	10	2.35	0.76	3.5	0.965	0.675
0.005	7	1.80	0.59	3	0.940	0.670
0.0025	5	1.07	0.16	0.7	0.970	0.766

* x_0=without plasma, x=with plasma.

** calculated from $E_f=\frac{x}{k}$, by the aid of Schutz' rule. k=0.219.

*** $i=1-\frac{E_f}{E}$, $i'=1-\frac{x}{x_0}$.

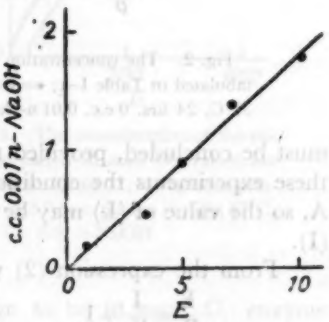

Fig. 1. The amount of hydrolysis and the enzyme concentration. —, calculated from k 0, 181, •, observed; 37°C, 24 hrs.; Increased acidity in 2 c.c. digestate., c.c. 0.01 n-NaOH.

Accordingly x_0-E curve describes a parabola of 1/2 order (Fig. 2).

On the other hand, in presence of the serum the amount of hydrolysis x is in direct proportion to the enzyme concentration.

From these equations

$$x_0=K\sqrt{E} \quad \ldots\ldots\ldots\

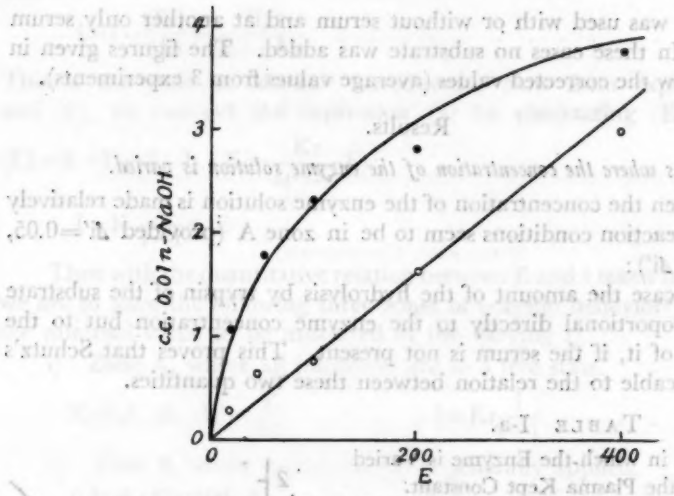

Fig. 2. The concentration of the enzyme is varied (Zone A). Values are tabulated in Table I–a; •=without plasma, ○=with 0.5 c.c. plasma (1:40); 37°C, 24 hrs. 0 c.c. 0.01 n-NaOH.

must be concluded, provided that K and K′ are arbitrary constants. In these experiments the condition of the reaction is thought to be in zone A, so the value of (I_f) may be regarded as approximately equal to that of (I).

From the expression (2) we can have

$$\frac{E}{E_f}=\frac{I}{K_I}+1$$

This together with expression (7)′ allows us to express the relation between E_f and x, in

$$\frac{E_f}{x}=\frac{K_I}{(K_I+I)K'}$$

As (I) can be made constant in a series of experiments and K′ and K_I are both arbitrary constants, when

$$\frac{K_I}{(K_I+I)K'}=K''$$

$$\frac{E_f}{x}=K'' \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (7)$$

This indicates that x is proportional to the concentration of the free enzyme (E_f). Now provided K=K″ for simplification, we can obtain the value of (E_f) by calculation, and in consequence the value of i, the inhibition coefficient which should be defined by the equation $i=1-\frac{E_f}{E}$.

These values just mentioned are according to the computation found constant irrespective of the concentration of the enzyme (Table I), which is consistent with the theory given in the equation (4), i.e. the reaction conditions should be in zone A.

Similar experiments with serum albumin produce the same results (Table I-b).

TABLE I-b.

Experiments with Serum Albumin.

2.0 c.c. 2 % casein+2.0 c.c. enzyme+2.0 c.c. 2 % serum albumin (E. Merck)-buffer solution or 2.0 c.c. buffer; Control: without substrate, corrected values presented.

E×10⁴ Relat. conc. of enz. %.	Acidity increased, c.c. 0.01n-NaOH*		Inhibition coefficient
	x_0	x	i**
0.01	1.85	0.45	0.976
0.005	1.49	0.22	0.976
0.0025	0.94	0.20	0.952
0.00125	0.64	0.17	0.930

* x_0 = without albumin, x = with albumin.
** calculated from k=0.191.

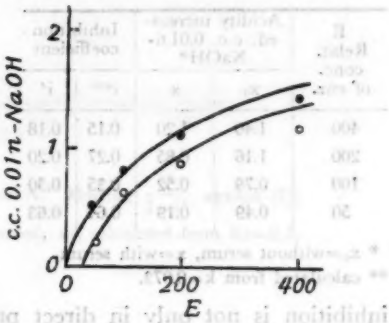

Fig. 3. The concentration of the enzyme is varied in zone C; values are tabulated in Table II; •=without plasma, ○=with 0.5c.c. plasma (1:50); 37°C, 3 hrs.; c.c. 0.01 n-NaOH.

b) When the reaction conditions seem to be in zone C, enzyme solution of relatively high degree of concentration used ($E' < 500\,\Delta i'$).

The relation-curve between the amount of hydrolysis and the concentration of enzyme takes a shape like parabola, whether there is serum added or not (Fig. 3). In this case there exists an approximate coincidence between the two inhibition coefficients i′ and i which are obtained respectively from the retardation of the increase in acidity and from (E) and (E_f) by the aid of Schutz's rule (Table II). This proves Schutz's rule to be applicable even to such a system with serum in zone C. But the results of these experiments can not always satisfy the equation (5), because it seems that the experimental errors have a great effect on one hand and parts of the reaction conditions lie perhaps in zone B on the other hand.

II. Cases in which the amount of the inhibitor is varied, with the concentration of the enzyme solution kept constant.

In these experiments I treated, for convenience sake only those whose reaction conditions are in zone A. As the results reveal, the degree of

TABLE II.

Experiments in which the Enzyme Varied while the Plasma Kept Constant.

(2) Concentration of the enzyme relatively high.

4.0 c.c. 1% casein+2.0 c.c. enzyme+0.5 c.c. plasma (1:50) or 0.5 c.c. buffer; 3hrs.

E Relat. conc. of enz.	Acidity increased, c.c. 0.01 n-NaOH*		Inhibition coefficient	
	x_0	x	i**	i′
400	1.40	1.20	0.15	0.18
200	1.16	0.85	0.27	0.20
100	0.79	0.52	0.35	0.30
50	0.49	0.19	0.64	0.63

* x_0=without serum, x=with serum.
** calculated from k=0.073.

TABLE III.

Experiments in which the Plasma is Varied while the Enzyme Kept Constant. (Zone A).

2.0 c.c. 2% casein+2.0 c.c. 0.01% enzyme +(I) c.c. plasma (2−I) c.c. buffer; 24 hrs.

Acidity increased in 2 c.c. digestate; c.c., 0.01 n-NaOH.

x_0 without plasma	x, with (I) c.c. plasma, (I) c.c.				
	0.2	0.5	1.0	1.5	2.0
1.64	1.13	0.51	0.40	0.28	0.22
$i'=1-\frac{x}{x_0}$	0.31	0.69	0.76	0.83	0.87
$\frac{i'}{1-i'}$	0.45	2.25	3.15	4.9	6.7

inhibition is not only in direct proportion to the concentration of the inhibitor, but the increasing rate of the value of inhibition coefficient i′ (obtained from increased acidity) becomes the lower according as the concentration of the serum added becomes the smaller (Table III).

In order to inquire into this relation quantitatively I rewrite equation (3) as follows by the aid of equations (6) and (7) and eliminating (E_f), which value can be found from equation (1),

$$\frac{i'}{1-i'}=\frac{I_f}{K_I\sqrt{E}}+\frac{1}{\sqrt{E}}-1 \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (8)$$

but K=K″ for simplification.

As in zone A (IE) is too small, in comparison with (I), to be negligible, (I_f)=(I) and the preceding expression can be

$$\frac{i'}{1-i'}=\frac{I}{K_I\sqrt{E}}+\frac{1}{\sqrt{E}}-1 \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (9)$$

(E) can be made constant in a series of experiments, so a straight line must be drawn between the two values when $\frac{i'}{1-i'}$ is plotted on ordinate and (I) abscissa. Figure shows that the results from Table VI are in exact accordance with the above theory when enzyme concentration is near 0.01% (Fig. 4). Similar experiments with serum albumin produce the same result (Fig. 5). Though the value of (E) is to be determined only relatively, in case that of $\frac{1}{\sqrt{E}}-1$ is small enough to be graphically neglected as above, equation (9) can be further simplified.

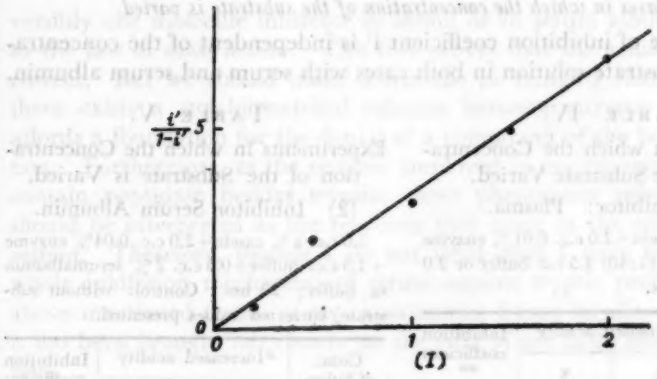

Fig. 4. The plasma is varied in zone A. Plotting $\frac{i'}{1-i'}$ against (I); Values are tabulated in Table III. observed, —, calculated from K_I=0.3, slope of the straight line.

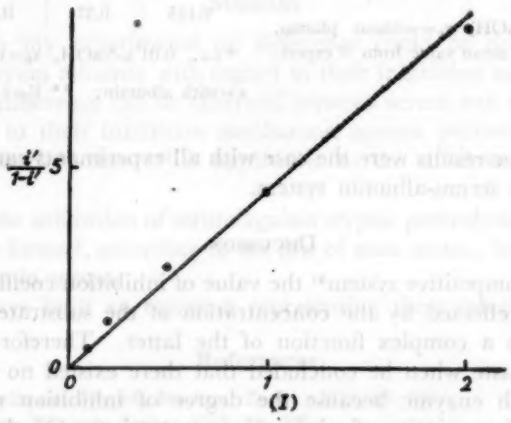

Fig. 5. Experiments with varying amount of serum albumin. 2.0 c.c. 2% casein+2.0 c.c. 0.01% enzyme+(I) c.c. 0.2 % serum albumin+2-(I) c.c. buffer; 37°C, 15 hrs., pH 8.0; formal-titration 2.0 c.c. digestate; •=observed, —=calculated from K_I=0.22.

$$\frac{i'}{1-i'}=\frac{I}{K_I} \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (10)$$

Thus the value of (I) as causing 1/2 inhibition (i'=0.5) may give a relative value to K_I.

III. Cases in which the concentration of the substrate is varied.

The value of inhibition coefficient i' is independent of the concentration of the substrate solution in both cases with serum and serum albumin.

TABLE IV.

Experiments in which the Concentration of the Substrate Varied.

(1) Inhibitor: Plasma.

2.0 c.c. a % casein+2.0 c.c. 0.01 % enzyme +0.5 c.c. plasma (1:40) 1.5 c.c. buffer or 2.0 c.c. buffer; 24 hrs.

Conc. of Substrate, %	*Increased acidity		Inhibition coefficient **
	x_0	x	
2	1.14	0.53	0.54
1	0.65	0.30	0.54
0.5	0.45	0.20	0.56
0.25	0.17	0.08	0.53
0.125	0.07	0.03	—

* c.c. 0.01 n-NaOH, x_0=without plasma, x=with plasma; mean value from 3 experiments.

** $i'=1-\frac{x}{x_0}$

TABLE V.

Experiments in which the Concentration of the Substrate is Varied.

(2) Inhibitor Serum Albumin.

2.0 c.c. a % casein+2.0 c.c. 0.04% enzyme +1.5 c.c. buffer+0.5 c.c. 2 % serumalbumin or buffer; 20 hrs.; Control: without substrate, corrected values presented.

Conc. of Substrate, %	*Increased acidity		Inhibition coefficient **
	x_0	x	
2	4.07	3.16	0.22
1	2.37	1.85	0.22
0.5	1.13	1.03	0.20
0.25	0.65	0.54	0.17
0.125	0.37	0.30	0.23

* c.c., 0.01 n-NaOH, x_0=without albumin, x=with albumin; ** $i'=1-\frac{x}{x_0}$

The above results were the case with all experiments carried out with regard to the serum-albumin system.

Discussion.

In the competitive system[3] the value of inhibition coefficient i should naturally be effected by the concentration of the substrate solution, for the former is a complex function of the latter. Therefore, Northrop[6] was in too haste when he concluded that there existed no compound of substrate with enzyme because the degree of inhibition might not be changed by the variation of substrate concentration. On the other hand, the classical theory that Michaelis and Menten[1] held would ascribe this unchangeability of the value of i to the fall of the splitting velocity of the substrate attacked by serum. But on such an occasion the value of inhibition coefficient can not be effected by the concentration of the enzyme, either. As a matter of fact, however, the result of my experiment was to the contrary.

Moreover, the result of my experiments coincides with the theoretical equation reached on the premise that one molecule trypsin combines re-

versibly one molecule inhibitor in serum or in serum albumin according to the law of mass action. This fact proves that the assumed premise is correct. But we should make a mistake in thinking that the fact that there exists a stoichiometrical relation between enzyme and inhibitor affords a foundation for the denial of a compound of the both by adsorption. Furthermore, as the enzyme employed in my experiments seems to contain peptidase besides trypsin, those phenomena referred to above should be interpreted as the reactions that come in the enzyme complex system. Therefore, though I do not think that I have made clear the whole inhibition mechanism of serum against tryptic proteolysis by the above mentioned results of my experiments, I may be allowed to say that it has been brought out clearly on the part of the molecular kinetics relation.

And Goldstein's theory[3] holds good not only for the substrate-trypsin-serum system, but in such cases where the amount of hydrolysis is not proportional to the concentration of enzyme. With respect to this relation I have succeeded in forming a theoretical expression.

Summary.

Through my experiments no difference could be noticed between serum and serum albumin with regard to their inhibition mechanism.

1. No difference can be observed between serum and serum albumin with regard to their inhibition mechanism against proteolysis.
2. Goldstein's theory[3] is applicable to the substrate-trypsin-serum system, too.
3. In the inhibition of serum against tryptic proteolysis the reversible compound is formed, according to the law of mass action, between enzyme and inhibitor in serum.
4. I have built an equation representing these relations.

References.

1) Michaelis and Menten, Biochem. Ztschr., 1913, **49**, 333.
2) Haldane, Biochem. Journ., 1915, **19**, 338.
3) Straus and Goldstein, Journ. Gen. Physiol., 1943, **26**, 559; 1944, **27**, 529.
4) Maruo and Koga, Journ. Jap. Chem., 1949, **3**, 25.
5) Northrop, Journ. Gen. Physiol., 1926, **9**, 767; 1932/33, **16**, 41.
6) Northrop, Journ. Gen. Physiol., 1922, **4**, 487; 1923, **5**, 335.

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Studies on Serum Antitrypsin.

Second Report.

A New Clinical Method by Formol-Titration of Measuring Antitryptic Index of Serum.

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Introduction.

The methods that have been adopted to clinically determine the antitryptic index of serum are all based on the dilution method, the values obtained by which are discontinuous. This dilution method is lacking in objectivity and fails in exactness with various other inconveniences. Previously I[1] studied the inhibition mechanism of serum against tryptic proteolysis from stand point of chemical kinetics, and now I must devise, on this theoretical foundation, a new simplified method which satisfies such a clinical request that only one drop of serum is sufficient for the purpose.

Experimental.

I. Theoretical Studies on the Determination on Antitryptic Index of Serum.

The following are the two methods theoretically conceivable, i.e.,

i) direct method: amount of the inhibitor in serum is computed directly from the value of the dissociation constant of the compound of enzyme with inhibitor, and

ii) indirect method: amount of the inhibitor in serum is known directly by using the value in proportion to it which has been obtained from the inhibition coefficient.

As the direct method, which Grob[2] tried refractometrically, it is pretty difficult to exactly determine the value of the dissociation constant; furthermore, when it varies, the method is very inconvenient to use. Therefore, I intend to treat the indirect method exclusively in my following studies.

If the reaction velocity at its beginning is proportional to the concentration of the enzyme, the inhibition coefficient, which is to be obtained from its retardation, can be found by using whatever value in proportion to the enzyme concentration and then Goldstein's equations[3] are perfectly available. In general cases, however, especially when the formol titration is employed, his equations are not serviceable without modifications because the acidity increased is not always proportional to the enzyme concentration. Of course, in these cases, too, the values propotionate to the enzyme concentration may be got by calculation without much difficulty, but experimental errors, here, have an effect powerful enough to make them useless. Accordingly it would be much better to use the following equation I[1] introduced.

$$\frac{i'}{1-i'}=\frac{(I)}{K\sqrt{E}}+\frac{1}{\sqrt{E}}-1 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

but (I) and (E) represent mol concentrations of all the inhibitor and all the enzyme respectively, while i' means the inhibition coefficient directly obtained from the retardation of the increased acidity. In equation (1) the reaction condition should be in zone A (so called by Goldstein[3]), so that the value of inhibition coefficient i, which has been got from the retardation of the initial velocity, can be given by his equations.

$$(I)=K_I\frac{i}{1-i} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Quantitative relations between i' and i are derived from (1) and (2) as follows,

i) $1>E$ . . . . . $i'>i$
ii) $1=E$ . . . . . $i'=i$
iii) $1<E$ . . . . . $i'<i$

As in zone A the value of i approximates to 1, only the third case ($i'<i$) is practically available. I determined such relations as above by the following experiments.

Method.

Reaction system: 2.0 c.c. 0.005—0.02 % pancreatin-m / 15-phosphate-buffer-solution (pH 8.0)+2.0 c.c. 2% casein-buffer-solution+0—2.0 c.c. plasma (1:40)+2.0—0 c.c. buffer-solution, incubation: at 37°C, 24 hrs. under toluol; acidity increased in 2 c.c. digestate measured by formol titration (0.01 n-NaOH).

Result.

The results of my experiments are given in Table I. A plot having $\frac{(I)}{i'}$ and $\frac{1}{1-i'}$ as ordinate and abscissa respectively produces a straight line

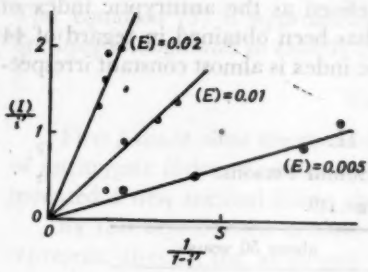

Fig. 1. Plotting $\frac{(I)}{i'}$ against $\frac{1}{1-i'}$ Values are computed from the value in Table I. $i'=\frac{x}{1-x_0}$; (E) %, relative concentration of enzyme.

TABLE I.

Antitryptic Activity of Blood Plasma When Varying its Quantity.

2.0 c.c. 2% casein+2.0 c.c. enzyme+(I) c.c. plasma+(2−I) c.c. buffer; 24 hrs.; 37°C.

Acidity increased in 2 c.c. digestate; c.c. 0.01 n-NaOH.

Relative conc. of enzyme %	x_0, without plasma	x, with (I) c.c. plasma				
		0.2	0.4	0.6	0.8	1.0
0.02	2.09	1.91	1.47	1.31	1.21	1.04
0.01	1.84	1.06	0.83	0.73	0.59	0.49
0.005	1.38	1.38	0.62	0.32	0.19	0.09

(Fig. 1), which coincides with the theory given in the equation (1). Practically, after a long while of digestion the value of intercept on the ordinate becomes negligible against that of $\frac{1}{1-i'}$ and we have a simplified equation,

$$\frac{(I)}{i'}=\frac{1}{1-i'}K_I\sqrt{E} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

In this case the value of i which has been drawn from the retardation of the initial velocity must be constant in zone A irrelatively to the enzyme concentration (2), but that of i' becomes again variable, even in zone A, as is given in the following equation as the quantitative relations.

$$i'=1-\frac{K_I}{K_I+(I)}\sqrt{E}=1-K\sqrt{E} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

From these results, experimental and theoretical, it is evident that the value of $\frac{i'}{1-i'}$ is in direct proportion to the amount of the inhibitor; therefore, this may be considered to represent the antitryptic activity of the serum.

When numeral cases are compared practically, it would be convenient if we should take the value of $\frac{i'}{1-i'}$ as approximately 1 for healthy persons. In order to answer this purpose the following conditions are concluded from the previous results:

Enzyme: 0.01%; Plasma (1:40) 0.5 c.c. (0.05 c.c. of blood which one drop contains is sufficient); Digestion hour; 24 hrs.

II. Clinical Determination.

When what is gained by multiplying the value of $\frac{i'}{1-i'}$ that has been

found as mentioned above by 100 is defined as the antitryptic index of serum, the mean value 119 (Table II) has been obtained in regard of 44 cases of healthy persons. This antitryptic index is almost constant irrespective of sex and age.

TABLE II.

Antitryptic Index of Sound Persons.

44 cases; average: 119.

below 35 years			above 50 years		
no.	male	female	no.	male	female
1	122	144	1	110	132
2	132	127	2	110	117
3	138	127	3	110	110
4	132	132	4	163	80
5	117	110	5	138	110
6	110	122	6	127	132
7	108	127	7	132	127
8	110	108	8	127	—
9	122	108	9	—	—
10	96	100	10	—	—
11	96	138	11	—	—
12	150	122	12	—	—
13	132	132	13	—	—
14	138	—	14	—	—
15	117	—	15	—	—
16	138	—	16	—	—
average	122	122	average	132	117

Total average: male 127 (24 cases), female 122 (20 cases); below 35 years 122 (29 cases), above 50 years 122 (15 cases).

DISCUSSION.

The antitryptic index thus obtained is in proportion to the amount of the inhibitor which is contained in 0.5 c.c. of blood plasma equivalent to 1/40 diluted blood. Such a value, too, is thought to indicate the antitryptic activity of serum even when the value of dissociation constant varies with the amount of the inhibitor constant. As the value of i′ varies according to the enzyme concentration as has been shown in the equation (4) though independent of the substrate concentration, the concentration of the enzyme needs to be kept constant in all possible cases. With this purpose in mind, we may practically pass without errors by having first an enzyme of higher concentration, then that of 0.01% through dilution

The equation (3) is approximately true only when the digestion hours are long enough and so exactly 24 hours must be prepared for digestion.

Conclusion.

First I made clear the theoretical foundation for the measuring method of antitryptic index of serum by formol-titration, then on this foundation invented a new method filling the clinical demand.

By this new-devised method we can obtain continuous values, which represent directly the antitryptic activity of serum.

References.

1) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 259.
2) Grob, Journ. Gen. Physiol., 1943, **26**, 405.
3) Goldstein, Journ. Gen. Physiol., 1943, **26**, 559.

The Tohoku Journal of Experimental Medicine, Vol. 53, Nos. 3 & 4, 1951.

Studies on Serum Antitrypsin.

Third Report.

Chemical Nature of Antitryptic Activity of Serum. I. Its Relation to Serum Protein.

By

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Introduction.

Neither substance theory nor physico-chemical theory[1] which have been established can decisively explain for the chemical nature of antitryptic activity of blood serum. Contrary to that the latter denies the special substance such as "antitrypsin", the earlier workers presumed protein[2] or lipoid substance[3] as inhibitor in serum. Although these lipoid and protein theories had been confronted with each other, today the latter being scarcely paid any attention to, the nature of "antitrypsin" should be mostly found in serum protein or in non-protein substance as protein split product[4] and polypeptide-like substances,[5] or in the both (dualistic theory, Utzkin-Ljubowzow)[6]. The well known fact that serum albumin inhibits trypsin action, can itself alone hardly explain the whole matter, as Oppenheimer[7] had already pointed out. However, no qualitative and quantitative differences were found between the inhibition mechanism of serum and that of serum albumin in the preceding experiment[8].

From such points of view to answer the question whether non-protein substance really exists in human serum as inhibitor or not, I intend in the first place to make clear the relation between inhibition activity of serum and serum protein.

Experimental.

Substrate: Casein (Hammarsten) or gelatin (Kokko); Enzyme: pancreatin (Iwaki), both resolved in m/15-phosphate-buffer solution (pH 7.8); Plasma: with 3.8% citrate diluted to 1:40.

The reaction mixture was incubated at 37° C under toluol; Increased

acidity in 2 c.c. digestate formol-titrated (0.01 n-NaOH) according to Northrop. For control, experiments without enzyme or without substrate in the presence of or in the absence of serum and an experiment with serum only and without enzyme nor substrate were carried on. Only corrected values are given in tables.

I. Tryptase activity of serum.

Method: Tryptase activity was examined with the globulin fraction obtained by the method of Rocha E. Silva.[9]

Results: After long digestion (72 hrs.) at pH 7.8, the hydrolysis of casein was, for the first time, clearly recognized.

TABLE I.

Tryptase Activity of Human Serum.

16 c.c. 2% casein (Hammarsten) + 4 c.c. globulin-buffer solution; pH 7.8, 37°C, increased acidity in 4 c.c. digestate, c.c. 0.1 n-NaOH.

Time in hrs.	Increased acidity
25	0.2 ; 0.15; 0.1 ; 0.15
72	0.2 ; 0.15; 0.15; 0.15
120	0.35; 0.45; 0.35; 0.35

TABLE II.

Protein Fraction of Serum and Antitryptic Activity.

4 c.c. 4% casein (gelatin) + 2 c.c. 0.01% enzyme + 1 c.c. globulin solution or buffer.

Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH							
Substrate	Time in hrs.	without globulin	with 1 c.c. globulin precipitated with Na_2SO_4-Aq. of molar concentration				
			1.5	1.25	1.0	0.75	0.5
casein	15	0.8	—	—	0.4	0.7	0.8
	24	1.25	—	—	0.95	1.2	1.25
gelatin	20*	0.8	0.45	0.5	0.6	—	—
	20	0.85	0.45	0.6	0.6	—	—
	20**	0.85	0.4	0.6	0.55	—	—

* with 0.5 c.c.; ** with 2 c.c. globulin solution.

II. Protein fraction of serum and antitryptic activity.

Method: By salting-out method with Na_2So_4, 0.5 c.c. serum was added with 7.5 c.c. Na_2So_4-solution of certain concentrations, after 30 min. at room temperature centrifugated; the precipitate washed twice with the same solution. The precipitate obtained for the last time resolved in 20 c.c. m/15-phosphate-buffer (pH 7.8) for the experiment (equivalent to 1:40 dil.).

Results: In the fraction which precipitated from over 1 mol. solution of Na_2So_4 inhibitory activity is evidently recognized, but not in that from under 0.75 mol. solution.

III. Antitryptic activity before and after the acid treatment.

Method: Acid treatment; Constant amount of serum after its pH value needed has been obtained with n/10-NaOH or -HCl, was incubated at

37°C. After the acid treatment its volume and its degree of dilution were made constant with buffer. For control to the acid-treated serum, the original serum was diluted to the same degree. For general control, reaction mixture without enzyme or substrate both with and without serum on one hand, and only with serum on the other hand under the same conditions was incubated. Corrected values are given in the tables.

Results: No change in antitryptic activity was seen with those which pH values had immediately been restored. After relatively long treatment of serum especially in those which have been treated under pH 4.0, disappears the antitryptic activity in proportion to the extent of the hydrolysis of serum protein by pancreas trypsin, which normally is hardly splitted by its action. Nevertheless, the appearance of the antitryptic activity was observed first in those which had been treated at pH rather more than 5.0, without any proportion to the latter and to the extent of the hydrolysis of serum protein.

Table III.

Antitryptic Activity Before and After the Acid Treatment.

2 c.c. 2% casein+2 c.c. 0.005 (0.01) % enzyme+0.2 c.c. serum (1:5)+2 c.c. buffer.

			Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH							
% Rel. conc. of enz.	Time of		with serum after the acid treatment at pH						without serum	with original serum
	digestion	acid treatment	1.3	3.5	4.0	4.5	5.0	6.0		
0.005	20	0	—	0.6	0.6	0.55	0.6	0.65	1.5	0.6
	20	20	—	0.9	0.8	0.6	0.55	0.5	1.4	0.55
0.01	10	0	0.1	—	—	—	—	—	0.95	0.1
	10	20	0.75	—	—	—	—	—	0.9	0.1

Table IV.

Hydrolysis of Serum Protein by Trypsin Before and After the Acid Treatment.

2 c.c. 0.1% enzyme+2 c.c. serum (1:5)+2 c.c. buffer.

Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH							
Time in hrs. of		Acid treatment at pH					
digestion	acid treatment	3.5	4.0	4.5	5.0	6.0	7.8
15	0	0.1	0.1	0.05	0.1	0	−0.05
	3	0.5	0.5	0.45	0.3	0.2	0.2

TABLE V.

Tryptase Activity of Serum Before and After the Acid Treatment.

2 c.c. 2% casein +0.5 c.c. serum (1:5)+4 c.c. buffer; pH 7.8.

Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH

Time in hrs. of		Acid treatment at pH						
digestion	acid treatment	3.5	4.0	4.5	5.0	6.0	7.0	7.8
24	0	—	—	0	0.1	0.15	0	0.05
	24	0	0	0.1	0.15	0.3	0.2	0.1
24	8	−0.05	0.1	0.1	0.15	0.2	—	—
15	0	0.05	0.05	0.15	0.2	0.15	—	0
24	24	0	—	0.05	0.25	0.3	−0.05	0

IV. Antitryptic activity before and after the adsorption with kaolin.

Method: pH values of serum were obtained in the same manner as above: a constant amount of serum with 2–3 c.c., 2–4% kaolin suspension was shaken for 30 min. and then centrifugated. After the centrifugation, the pH value of the supernatant was restored to 7.8 by adding buffer that its volume and concentration might be made constant.

Results: After the adsorptions, decrease in antitryptic activity is most evident when adsorption is carried out at pH near 4.6 and never seen at below 3.2 and above 5.4 (Table VI). Then antitryptic activity was examined of the precipitate that had precipitated at pH 4.6 and also of the supernatant, for an isoelectric point of a serum protein also shows the same pH value 4.6 approximately. The results show that the latter inhibits the trypsin action as to the same extent as the original serum, although the former inhibits a little. Accordingly the decrease in inhibitory activity as seen in Table VI must be due to the effect of the adsorption. Further the inhibitory activity can be perfectly diminished when adsorption was repeated three times at pH 4.6 (Table VIII). The amount of hydrolysis

TABLE VI.

Antitryptic Activity Before and After the Adsorption with Kaolin.

2 c.c. 2% casein+2 c.c. 0.01% enzyme+1 c.c. serum (1:50)+2 c.c. buffer.

Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH

Time in hrs.	with 1 c.c. serum adsorpted at pH							without serum
	3.2	3.8	4.2	4.6	5.4	6.2	(7.8)	
15	0.7	0.9	0.8	1.0	0.6	0.6	0.6	1.25
24	0.8	1.1	1.15	1.3	0.85	0.8	0.85	1.5

of casein in this case is bigger than that in control experiment without serum (Table VIII), which may be considered that the action of serum tryptase is summed together with that of pancreas trypsin in reaction mixture (Tables I & VI). Still the decrease in N-amount and that in inhibitory activity do not take place in parallel.

TABLE VII.

Antitryptic Actitivity of Serum.

4 c.c. 2% casein+2 c.c. 0.01% enzyme+1 c.c. serum (1:40); pH 7.8; 37°C; increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH

Time in hrs.	Precipitate*	Supernatant	Original serum	without serum
15	0.55	0.05	—	1.2
24	0.7	0.15	0.15	1.3

* resolved in buffer equivalent to 1:40 dilution of serum.

TABLE VIII.

Nitrogen Amount of Serum and Antitryptic Activity.

Adsorption with kaolin at pH 4.6; 4 c.c. 2% casein+2 c.c. 0.01% enzyme+1 c.c. serum (1:40); increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH.

Time in hrs.	without serum	with original serum	with serum after the adsorption		
			1st	2nd	3rd
15	1.5	0.5	1.25	1.45	1.55
40	2.0	1.2	1.25	2.1	2.4
N-amount, mgr/c.c.		0.61	0.21	0.16	0.07

* direct nesslerization method, (micro-Kjeldahl).

DISCUSSION.

To make an argument with serum "antitrypsin", whether tryptase in serum exists or not should be of importance. From the results above (Tables I, V & VIII) tryptase activity (casein splitting, pH 7.8) can be evidently recognized in serum though it generally is covered with inhibitor and not to be detected directly. It must according to Christensen[10] be studied as an enzyme "plasmin" distinguished from pancreas trypsin.

The inhibitory action of the protein farctions precipitated from Na_2SO_4-solution of 1.0–1.5 mol. concentration as already seen above, indicates that the antitryptic activity of serum must be attributed not only to serum albumin, which is generally accepted, but also to globulin fraction. It has not yet been decided whether the antitryptic activity of serum should be directly attributed only to serum protein itself, or otherwise a wide existence of an antitryptic base or substance on protein molecule of each fraction should be expected. This is interesting to me, for the serum proteins themselves are usually resistent against pancreas trypsin.

From the experimental results by the acid treatment, it is evident that it requires a relatively long time for the acid treatment, to diminish the inhibitory activity of serum, and that the smaller is the pH value employed

for the treatment indifferently from the optimal pH value for catheptic enzyme action, the more evidently disappears the antitryptic activity in proportion to the extent of the hydrolysis of serum protein by pancrease trypsin which seems resulting from degeneration of it by the acid treatment. This fact says that the disappearance of antitryptic activity occurs in parallel to the degree of the degeneration of serum protein. This is analogous to the .act shown by Chrometzka,[1] that the serum had lost its antitryptic activity in proportion to the change in viscosity by acid treatment, and upon that he asserted his physico-chemical theory. On the other hand, provided the inhibitor might be destroyed at pH 4.5 by some substance as stated by Widmark[11] it should not be attributed to the action of the catheptic enzyme in serum. And the appearance of the serum tryptase activity when it was treated at above pH 4.8 is imagined to be the consequence of the enzyme itself having also been degenerated under pH 4.5 through the treatment. In addition to these, the results from the experiments on adsorption elucidate that the optimal pH value for the disappearance of the inhibitory action coincides nearly with isoelectric point of serum protein. All of these above mentioned facts are indicating that inhibition has some connections with the serum proteins, but now it must be attended to that the decrease in N-amount through adsorption does not always proportional to that in inhibitory action of serum, and moreover that the antitryptic activity is observed also with globulin fraction besides albumin fraction.

SUMMARY.

1. Tryptase activity in serum (pH 7.8, hydrolysis of casein) is generally inhibited by serum itself and is not to be recognized directly.

2. The inhibitory action against pancreas trypsin can be demonstrated not only in albumin fraction but also in globulin fraction of serum.

3. The smaller the pH value (under pH 4.2) employed for the acid treatment is, the more evident is it that the antitryptic activity of serum after the treatment disappears in proportion to the degree of degeneration of serum protein. The appearance of serum tryptase activity is distinct after the treatment rather at more than pH 4.8.

4. After the adsorption with kaolin at nearly isoelectric point of a serum protein, antitryptic activity of serum disappears not proportionally to the decrease in N-amount of the serum.

References.

1) Chrometzka, Ztschr. ges. exp. Med., 1930, **69**, 665; 1932, **80**, 395.
2) Hedin., Journ. Physiol., 1905, **32**, 390; etc.
3) Jobling and Petersen, Journ. Exp. Med., 1914, **19**, 459; etc.

4) Northrop, Journ. Gen. Physiol., 1924, **4**, 227.
5) Northrop, Science, 1935, **81**, 418; Journ. Gen. Physiol., 1936, **19**, 991; Schmitz, Ztschr. pyhsiol. Chem., 1938, **255**, 234; Kunitz, Journ. Gen. Physiol., 1947, **30**, 311.
6) Utzkin-Ljubowzow, Biochem. Ztschr., 1928, **194**, 296.
7) Oppenheimer, Ferment u. ihre Wirkg., II vol., 1926, 5ed. 1089.
8) Hayakawa, Tohoku Journ. Exp. Med., this volume, 243 and 251.
9) Silva, Biochem. Journ., 1948, **43**, 163.
10) Christensen et al., Journ. Gen. Physiol., 1945, **28**, 559.
11) Widmark, Ztschr. physiol. Chem., 1932, **207**, 182.

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Studies on Serum Antitrypsin.

Fourth Report.

Chemical Nature of Antitryptic Activity of Serum. II. Its Symplextheory.

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In the previous report[1] I demonstrated that the inhibitory effect of serum upon tryptic proteolysis was in close connection with serum protein, pointing out a few results that should be attended to. In the present paper, I deal further with the experiments on kaolin adsorption.

Experimental.

Method.

Adsorption with kaolin: the same in the previous paper; elusion: the kaolin adsorbate was eluted with m/15-phosphate-buffer (pH 7.8) letting its volume diluted to be equivalent to 1:20 serum at 37°C for 24 hrs.; For control, original serum diluted with buffer (1:20); N-metry: by means of direct nesslerization method according to Ssokolnikow[2] (micro-kjeldahl).

Results.

After the adsorption at pH 3.2 or 5.6 by which no decrease in inhibitory action might be expected as seen in the previous paper,[1] amount of N decreases to nearly one third of original serum, its antitryptic activity not being affected. Although amount of N decreased nearly one fifth of original serum, after two adsorptions at pH 3.2 and 7.2, yet the antitryptic activity was scarecely affected. Moreover, after adsorption was conducted twice at pH 3.4, the latter became rather larger than ever, in spite of the distinct decrease in amount of N in serum.

To examine the quantitative relation between the adsorpted serum with higher inhibitory activity despite of its lower content of N, and its degree of inhibition, the experiments took place, in which the serum was

TABLE I.

Nitrogen Amount of Serum and Antitryptic Activity.

4 c.c. 1% casein+2 c.c. enzyme+1 c.c. serum or buffer; pH 7.8, 37°C; serum: 1:40 dil. Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH.

% Relat. conc. of enzyme	Time in hrs.	without serum	with original serum	with serum adsorpted at pH	
				3.2	5.6
0.01	17	0.85	0.30	0.30	0.35
	20	1.05	0.35	0.35	0.40
0.005	17	0.65	0.20	0.15	0.15
	20	0.75	0.25	0.25	0.20
N-amount, mgrm./c.c.			0.95	0.35	0.32

TABLE II.

Nitrogen Amount of Serum and Antitryptic Activity.

4 c.c. 2% casein+2 c.c. 0.01% enzyme +1 c.c. serum (1:40) or buffer; pH 7.8, 37°C; Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH.

Time in hrs.	without serum	with original serum	with serum adsorpted at pH	
			3.2	3.2&7.3
4	0.95	0	0.05	0.25
20	2.75	0.40	0.40	0.60
N-amount, mgrm./c.c.		0.95	0.95	0.17

TABLE III.

Nitrogen Amount of Serum and Antitryptic Activity.

4 c.c. 2% gelatin+2 c.c. enzyme+1 c.c. serum (1:40); pH 7.8, 37°C; Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH.

% Relat. conc. of enzyme	Time in hrs.	without serum	with original serum	with serum adsorpted at pH 3.4
0.01	2	0.40	−0.05	0
	20	1.90	0.65	0.40
0.005	2	0.15	0.15	0.05
	20	0.30	0.45	0.25
N-amount, mgrm./c.c.			0.82	0.42

varied, while the enzyme and the substrate kept constant. The results show that the relation curve between these two quantities gets nearer to a straight line according as the N-amount decreases, although the curve with the original serum draws sigmoid form. Also in such a case the increase in antitryptic activity of some serum adsorpted with kaolin, is found despite of its lower content of N, as is seen in Table III, which seems resulting from the change of inhibition mechanism.

Now an attempt to get the antitryptic activity, which was once diminished after adsorption, again from the adsorbate, was achieved only

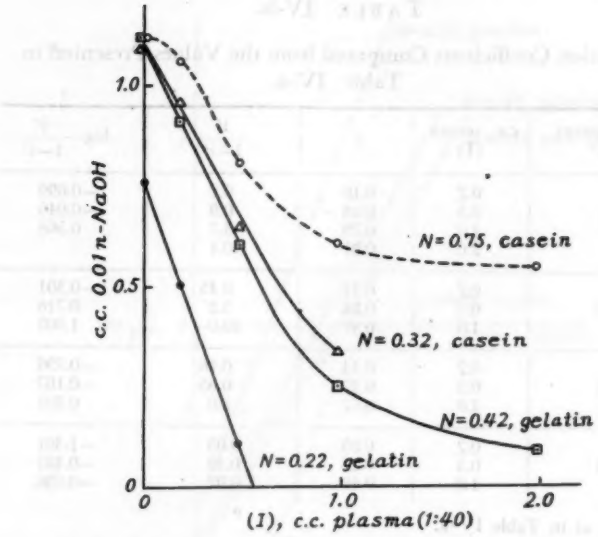

Fig. 1. Plasma (Serum) is varied. Values are tabulated in Table 4–a, c.c. 0.01 n-NaOH; N N-amount, mgrm./c.c.; Digestion, at 37°C, 20 hrs, pH 7.8; —, with serum (plasma) adsorpted with kaolin at pH 3.0–3.2; . . . , with original serum (plasma).

TABLE IV-a.

Experiments in which the Serum is Varied While the Enzyme Kept Constant.

4 c.c. 2% substrate-solution+2 c.c. 0.01% enzyme+a c.c. serum (1:40)+ (2–a) c.c. buffer; adsorption with kaolin: at pH 3.2–3.0, for several times; digestion: pH 7.8, 37°C.

Experiment no.	Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH								N-amount, of serum, mgrm./c.c.
	2% Substrate	Time in hrs.	without serum	with a c.c. serum, a c.c. added					
				0.2	0.5	1.0	1.5	2.0	
1	Gelatin	20	1.10	0.90	0.60	0.25	—	0.10	0.42
2		20	0.75	0.50	0.10	0	0	—	0.22
3		14	1.40	—	—	0.40	—	0.35	0.82*
4	Casein	20	1.10	0.95	0.65	0.35	—	—	0.32
5		20	1.10	1.05	0.80	0.60	0.55	—	0.75*

* Original serum without adsorption.

TABLE IV-b.

Inhibition Coefficients Computed from the Values Presented in Table IV–a.

Experiment no.*	c.c., serum (I)	i′	$\frac{i'}{1-i'}$	$\log\frac{i'}{1-i'}$
1	0.2	0.18	0.2	−0.699
	0.5	0.43	0.9	−0.046
	1.0	0.79	3.7	0.568
	2.0	0.91	10.1	
2	0.2	0.31	0.45	−0.301
	0.5	0.84	5.2	0.716
	1.0	0.97	32.0	1.505
4	0.2	0.14	0.16	−0.796
	0.5	0.39	0.65	−0.187
	1.0	0.67	2.0	0.301
5	0.2	0.05	0.05	−1.301
	0.5	0.25	0.33	−0.481
	1.0	0.48	0.92	−0.036

* The same as in Table IV–a.

TABLE V.

Experiments with Eluate (1).

2 c.c. 2% casein+2 c.c. enzyme+2 c.c. eluate or buffer; pH 7.8, 37°C. Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH. (24 hrs.)

% Relat. conc. of enz.	without serum or eluate	with original serum	with eluate
0.01	2.10	0.35	1.15
0.005	1.10	0.10	0.55
0.0025	0.60	0.10	0.35
0.00125	0.50	0.05	0.20

TABLE VI.

Experiments with Eluate (2).

3 c.c. 2% casein+2 c.c. enzyme+a c.c. eluate+(2–a) c.c. buffer; pH 7.8, 37°C. Increased acidity in 2 c.c. digestate; c.c. 0.01 n-NaOH.

% Relat. conc. of enz.	Time in hrs.	without eluate	with a c.c. eluate		
			0.5	1.0	2.0
0.01	16	1.50	—	—	0.90
	24	2.25	—	—	1.00
0.005	16	1.35	1.20	0.90	—
	24	1.80	1.25	0.85	—

to some extent. Also in this case the quantity of the eluate is nearly proportional to its degree of inhibition in the range of this experiments.

Discussion.

Formerly Utkin-Ljubowzow[3] demonstrated that the Na_2HPO_4-eluate from the colloid iron adsorbate of serum albumin solution had strong inhibitory activity (N-amount: 0.21 mgrm./c.c.). Recently it was found by Matsuo[4] that the serum albumin no more inhibited the pancreas

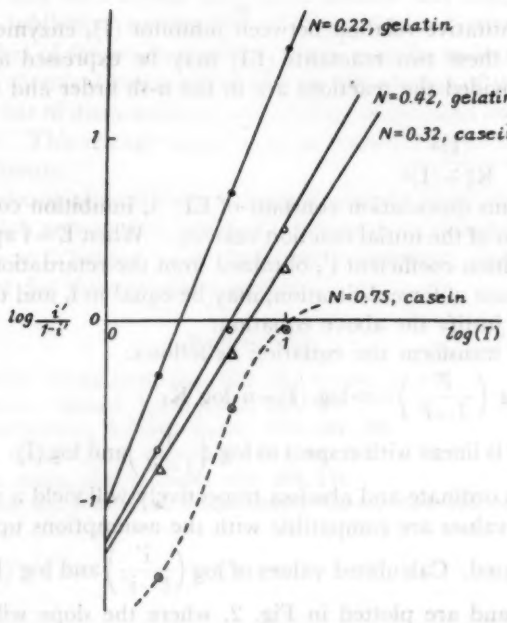

Fig. 2. $\log\frac{i'}{1-i'}=$n. log (I)−n log K_I Values are tabulated in Table IV–b.

trypsin action when it had been dialyzed. And as shown above, owing to the decrease in N-amount, the inhibition mechanism may undergo some change, which is similar to the fact which A. Schmitz[5] had observed with polypeptide-like inhibitor obtained from a bull blood plasma. In view of these respects the existence of some nonprotein inhibitory substance in human serum may be presumed. Provided such an inhibitory substance really is found in human serum, as antitryptic activity always disappears when N-amount becomes below 0.2 mgrm./c.c., it may be an N-compound, e.g., such polypeptide as Schmitz's[5] which is probably produced as protein split product in human body.

However, the facts above stand right opposite to that in the previous paper,[1] which is unable to give any solution either for the confrontation of protein and non-protein theory or for that of these and physico-chemical theory.

Symplex theory for the antitryptic activity of serum.

In the following I intend to study from the standpoint of chemical kinetics, how the change in the inhibition mechanism as above might be

brought forth.

The quantitative relation between inhibitor (I), enzyme (E) and the compound of these two reactants (EI) may be expressed approximately as follows, provided the reactions are in the n-th order and (I) ≪ (E) for simplification:

$$i=\frac{(I)^n}{K_I^n+(I)^n}$$

where K_I means dissociation constant of EI: i, inhibition coefficient from the retardation of the initial reaction velocity. When E=1 approximately, another inhibition coefficient i′, obtained from the retardation of increased acidity by means of formol-titration, may be equal to i, and then the value of i′ will also justify the above equation.

We may transform the equation as follows:

$$\log\left(\frac{i'}{1-i'}\right)=n\cdot\log(I)-n\cdot\log K_I$$

This equation is linear with respect to $\log\left(\frac{i'}{1-i'}\right)$ and $\log(I)$. A plot having these terms as ordinate and abscissa respectively will yield a straight line if the observed values are compatible with the assumptions upon which the equation is based. Calculated values of $\log\left(\frac{i'}{1-i'}\right)$ and $\log(I)$ are given in Table IV–b and are plotted in Fig. 2, where the slope will numerically equal n and the abscissa intercept will equal $\log K_I$. From this Fig. 2 it becomes clear that the smaller the N-content is, the larger becomes the values of n and the smaller that of K_I, indicating that the number of inhibitor-molecules combining with one molecule enzyme is increasing while the values of K_I is decreasing gradually with the drop of N-content. In such a case the treated serum of lower content in N will compatibly give much higher inhibitory activity than ever as seen above, which is hardly explainable by the classical theories. In this respect, therefore, for the chemical nature of antitryptic activity of serum my symplex theory may be established under which some inhibitory non-protein substance (probably polypeptide-like one) as prosthetic group on one hand and serum protein as "Träger"[8] on the other hand for "antitrypsin" of serum like those for enzyme structure are considerable. With such symplex theory all the results and theories already mentioned are compatibly understood, because the change of the inhibition order, or that of dissociation constant and thus that of inhibition mechanism, all of these might resulting from the changes of physico-chemical conditions of serum protein.

Summary and Conclusion.

1. After the kaolin adsorption at pH below 3.2 the serum with lower

content of N and with strong antitryptic activity was obtained.

2. The inhibition mechanism of thus obtained serum apparently differs from that of the original serum, which may be understood from the standpoint of chemical kinetics as based on the change of the order of inhibition and that of dissociation constant of the compound between enzyme and inhibitor. This change seems to be in consequence of the decrease in N-amount of serum.

3. For chemical nature of antitryptic activity of serum this symplex theory, in which non-protein inhibitory substance as prosthetic group and serum protein as "Träger" are considered respectively, is conceivable as similar to that for the enzyme structure.

References.

1) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 278.
2) Ssokolnikow, Biochem. Ztschr., 1927, **213**, 414.
3) Utkin-Ljubowzow, Biochem. Ztschr., 1928, **194**, 296.
4) Matsuo, Tohoku Journ. Exp. Med., 1941, **41**, 108.
5) Schmitz, Ztschr. physiol. Chem., 1938, **255**, 234.
6) Willstätter and Rohdewald, Ztschr. physiol. Chem., 1934, **225**, 103.

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Studies on Serum Antitrypsin.

Fifth Report.

Serum Antitryptic Index of the Patients with Castric or Duodenal Ulcer and with Gastric Cancer.

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Introduction.

With regard to the formation of ulcers Bordyreff[1] and Stuber[2] separately gave their opinions that these phenomena are all of them caused by the tryptic action of reversed duodenal juice and so should be called "tryptisches Ulkus", though peptic ulcers have been the common term.

Raab[3] attributes to trypsin the direct cause there being very rare cases of cancers in comparison with duodenal ulcers.

With the theory of inhibition mechanism as the basis instead of the existing process I[4] have developed a new method measuring of the antitryptic index of serum by formol-titration, succeeding in comparing the magnitude of values directly in a great many cases. It seems very interesting to attain to a precise knowledge by this method in view of the various opinions mentioned above and furthermore, this knowledge will serve a great deal in the diagnosis and importance of the antitryptic activity of serum.

By the by, in this paper the term antitrypsin means the inhibition activity of serum against pancreas trypsin.

Experimental.

Measuring Method and Results.

The method used was what I invented. When the question of time-lapse was handled the mensuration was taken at intervals of 10–14 days, whenever possible.

1. *Antitryptic index of sound persons.*

The mean value 108 (52) was obtained from 11 cases of sound people.

In each case the value was nearly constant, giving no change by the lapse of time. The figure in the parentheses shows the rate of inhibition (%), so with the following cases.

TABLE I-a.

Antitryptic Index (AT-Index) of Healthy Persons (1).

11 cases, male and female; average 108 ($i'=52$).

no.	$\frac{\%}{i' \times 100}$	AT-Index $\frac{i' \times 100}{1-i'}$
1	58	138
2	58	138
3	50	100
4	48	92
5	47	88
6	47	88
7	52	108
8	47	88
9	54	117
10	59	144
11	51	104

TABLE I-b.

Antitryptic Index of Healthy Person (2), During the Lapse of Time.

(30 years old, male, healthy)

Date	17/IV	10/V	14/VI	25/VI
$i' \times 100$	54	54	54	52
$\frac{i' \times 100}{1-i'}$	117	117	117	108

TABLE II.

AT-Index and Eosinophilia by Ulcer Patients

Eosinophilia *	Number of cases	Variation of AT-Index 0–40	41–60	61–100	over 101
(+)	22	7	3	7	5
(+)	9	7	2	0	0

* (—) below 800, (—) over 801 in 1 rm^3 blood respectively.

2. *Antitryptic index of gastric or duodenal ulcer cases.*

When 44 patients with a gastric or a duodenal ulcer were examined, the result was that though some gave a remarkably smaller value than a well person, the average value was 137 (57.9) a little bigger than the normal value of healthy men, 108 (risk rate: 1.0%). 29 cases out of 44 giving the figure of more than 122 (55), while we had only 3 cases in the examination of 11 sound persons (risk rate: 4.2%). There was no difference found between the cases of gastric ulcer and those of duodenal ulcer. This experiment brings out the fact that generally speaking the greater number of ulcer cases have the normal value or a value a little higher than that, which, on the whole, corresponds with the result of Chrometzka[5] who tried experiments viscosimetrically upon many patients.

Next I examined the change of antitryptic index by observing the clinical progress. When the examinees were with eosinophilia, the number of those whose antitryptic index varied by more than 41 was smaller than when they were without it (risk rate: 1.5%).

As to the form of niche seen when x-ray examinations are made of

gastric ulcer the tent-type or the finger-trace-type are superior in numbers among those showing the variation of over 61 and the flower-bed-type, among the others (risk rate: 0.4%). This also true when same investigations are carried out after the examined objects have been divided into two classes with a variation of 31 as the border line. The tent-type or the finger-trace-type are also predominantly seen among those having antitryptic index of more than 166 during the lapse of time, while the flower-bed-type, among those with that of less than 165 (risk rate: 0.6%). The former proves the acute process, the latter the chronic process. However, the position of niche, its size and the change of clinical findings showed no relation to the value of antitryptic index.

TABLE III.

AT-Index and Type of Niche by X-ray Examination.
(17 gastric ulcer cases).

Type of niche *	Variation of AT-Index				Maximal value of AT-Index		
	0–30	31–60	61–100	> 101	< 165	166–200	> 201
I II⇆I	0	0	3	3	0	1	6
II	0	1	1	1	1	0	1
III III⇆II	6	1	1	0	6	0	1

* I, Tent-type; II, Finger-trace-type; III, Flower-bed-type; ⇆ changing type.

TABLE IV.

AT-Index of the Patients with Duodenal Ulcer and Their Clinical Progress.

Clinical progress *	Number of cases	Variation of AT-Index	
		below 40	over 41
cured or improved	8	7	1
stationary or deteriorated	6	1	5

With regard to cases of duodenal ulcer, those whose clinical progress is favorable are found chiefly among those having the variation of less than 40, while patients of more than 41 make, many of them, unsatisfactory progress (risk rate: 2.4%). In these x-ray examinations in 1 case out of 3, when they had the variation of more than 61 and in 8 cases out of 11, whose variation was less than 61, pockets were revealed.

Next, out of 9 patients who underwent an operation to remove the ulcer affected parts, 4 had the antitryptic index rise after the operation and then restored again, while 5 made no change (variation less than 40) of their indices. The relation between those two is nearly the same when operations have not been performed. Thus it is thought there can be no particular effect produced by the removal of affected parts.

3. *Antitryptic index of gastric cancer.*

The following is the result of my observation made of 30 cases of gastric cancer through their clinical progress.

Among those patients who showed no change of antitryptic index (variation below 60) in their clinical progress or who had always the index of not less than 161 at the lowest, more were found with ascites than among those who showed some changes or whose antitryptic index was sometimes smaller than 160 (risk rate: 0.1 and 0.6% respectively). I further found more with more than (+) degrees of cachexia among the former (risk rate: 0.6 and 1.1% respectively) and also more with above (++) degrees of the cachexia than among the latter (risk rate: 0.6%).

TABLE V.

AT-Index and Ascites. (31 gastric cancer cases).

Ascites*	Variation of AT-Index				Minimal value of AT-Index		
	0–30	31–60	61–100	over 101	below 160	161–200	above 201
(+)	6	3	1	3	3	3	6
(−)	4	2	7	5	14	3	1

* Biopsy finding.

TABLE VI.

AT-Index and Degree of Cachexia. (33 gastric cancer cases).

Degree of cachexia*	Variation of AT-Index				Minimal value of AT-Innex		
	0–30	31–60	61–100	over 101	below 160	161–200	above 201
(−)	1	0	4	2	6	1	0
(+)	3	1	3	3	7	1	2
(++)	3	3	0	0	3	1	1
(+++)	3	1	1	2	1	3	4

* Classified as follows:
1. red blood corpsular below 2.5 mil. or Hb-content (Sahli) below 30%, 2. loss in weight within 2 weeks before the operation over 2 kg., 3. existence of edema or of ascites etc.; each calculated as (+), the number of (+) are given as degree of cachexia.

Next, many cases of peritonitis were discovered among those with the variation of below 70 or those whose lowest index was always above 161, when compared with the classes of more than 70 of variation of sometimes below 160 of index (risk rate: 2.4 and 0.2% respectively). In these experiments I knew that the difference in the highest values or that in the average values had no relation to the above questions.

In 17 people whose affected parts with cancers seemed to have been removed by gastrectomy and 8 people who only underwent the operation of gastroenterostomia, 2 and 3 respectively showed the rise of antitryptic index after the operation, while 8 and 1 respectively had their indices fall (each by more than 51). Though it seems that the cases belonging to the

TABLE VII.

AT-Index and Peritonitis by Gastric Cancer (30 cases).

Peritonitis*	Variation of AT-Index		Minimal value of AT-Index		
	below 70	over 71	below 160	161–200	over 201
(+)	9	2	2	4	5
(−)	7	12	15	2	2

* Biopsy finding.

falling class are comparatively numerous among those who had gastrectomy, the numbers of 7 and 4 respectively that remained without changes (variation below 50) show that there is scarecely any difference between the two kinds of the examined cases. The amount of metastasis, the degree of adhesion and that of lost weight bear no relation to the value of antitryptic index.

Through the above examinations there is no immediate connection found between the value of antitryptic index, its change, results of laboratorical examinations (i.e. blood sedimentation rate, acidity of gastric juice, hematological findings and blood pressure).

4. *Comparison between the antitryptic index of gastric ulcer and that of gastric cancer.*

The Table VIII shows the various average values measured of 31 ulcer cases and 30 cancer cases. The total average of cancers is high in comparison with that of ulcers (risk rate: 0.1%). Those whose antitryptic index goes above 186 (66) in their clinical progress are many among the sufferers with a cancer (risk rate: 0.1%). If the variation of 60 is taken

TABLE VIII.

Average Values of AT-Indices Measured of 31 Ulcer and 30 Cancer Cases.

Diagnosis	Varying					Non-varying		Total	
	Number of cases	AT-Index				Number of cases	AT-Index	Number of cases	AT-Index
		max. value	min. value	mean value	variation				
Gastric ulcer	9	218	109	154	109	8	134	17	144
Duodenal ulcer	3	233	111	157	124	11	124	14	131
Ulcer (total)	12	222	110	155	113	19	128	31	138
Gastsic cancer	15	270	147	194	114	15	195	30	195

as the border line and all the patients of both kinds are divided into the class of changing and non-changing, the highest, the lowest, and the average value of cancer cases are respectively bigger than those of ulcers in the changing group (risk rate: 2, 4.9 and 1% respectively). As regards the distribution condition there are also found more cases with cancer than those with ulcer that have more than 218 as the highest value, more than 110 as the lowest and more than 155 as the average (risk rate: 1.5, 2.3 and 1.1% respectively).

TABLE IX.

Distribution of AT-Indices of Patients with Ulcer or Cancer.

Diagnosis	Number of cases	AT-Index 0–122	123–185	186–300	above 301
Ulcer	43	14	20	9	0
Cancer	35	4	11	17	3

TABLE X-a.

Distribution of AT-Indices of Varying Class.

(1) Maximal value.

Diagnosis	Number of cases	AT-Index 0–217	218–270	over 271
Ulcer	9	5	2	2
Cancer	15	1	9	5

TABLE X-b.

(2) Minimal value.

Diagnosis	Number of cases	AT-Index 0–109	110–147	above 148
Ulcer	12	7	4	1
Cancer	15	3	5	7

TABLE X-c.

(3) Mean value.

Diagnosis	Number of cases	AT-Index 0–154	155–194	above 195
Ulcer	9	7	0	2
Cancer	15	3	6	6

TABLE XI.

Direction of Varying. (AT-Index of Varying Class)

Direction of varying	AT-Index cancer below 194	cancer above 195	ulcer below 149	ulcer above 150
Rising	5	2	5	1
Falling	0	8	1	5

TABLE XII.

Distribution of AT-Indices of Non-varying Class. (Mean value).

Diagnosis	Number of cases	AT-Index 0–134	135–150	151–195	above 196
Ulcer	8	4	3	1	0
Cancer	15	1	3	3	6

Furthermore, in the changing group of cancers, among those whose antitryptic index is less than 194 there are few who make their falling way by comparison with those taking their way upwards. On the contrary, among ulcer sufferers of this group with an antitryptic index of more than

150, the rising are few in comparison with the falling (risk rate: 1.0 and 4.2% respectively). In both cases another value was taken is the border and the condition of rising or falling was examined. The result was that there was no distinct difference. In addition, in the changing class the degree of change is almost the same with both cases.

As for the non-changing class, cancer cases are bigger in their mean value than ulcers (risk rate: 2%) and the former also include more cases in the distribution who show more than 151 and more than 135 respectively of mean value (risk rate: 0.9 and 2.9% respectively).

With all the above results in view, the values of inhibition rate, though to some extent they can tell the same relation or tendency mentioned above, will be in danger of not realizing satisfactory results because of their frequency where the difference can not be distinguished.

Discussion.

1. *The nature of antitrypsin.*

Previously I[6] stated that it was caused by the changes of inhibition mechanism that as we removed serum protein by adsorption, in some cases antitryptic index grew rather big and advocated the "symplex theory" of antitrypsin. The results of my experimental studies proved that as to cancer sufferers there were many who had a big and lasting antitryptic index among those with cachexia of high degree and that as to ulcer patients many of those having a big index of antitrypsin showed acute process. This corresponds with the long-existing view that when the destruction of protein of the body is in great activity, antitryptic index increases in its value. With these facts taken into consideration, we may conclude that antitryptic index generally becomes big when amount of serum protein (especially that of albumin) decreases.

According to my symplex theory[6] a single factor does not always cause antitryptic index to change; the quantitative and qualitative changes of an inhibitor and "Träger" may be each the factors. For example, even when the amount of the inhibitor does not change, yet the change of dissociation constant of its compound with the enzyme or the orderly change in n-th order inhibition may make antitryptic index big. Therefore, we need not seek the cause of the increase of antitryptic index, from common sence, only in the increased split product of tissue protein.

Furthermore, as these phenomena seem to be easily caused by the change of physico-chemical conditions of serum protein as "Träger", the pending questions between the two opposite theories, physico-chemical and stuff are smoothly resolved by this symplex-theory.

2. *Signification to living things.*

From the foregoing results the change of antitryptic index is understood as a biological reaction against the pathological process of living beings and also as the defensive mechanism against the protein destruction by tryptase, pancreas included, from a teleological point of view. Accordingly it is presumed that antitryptic index is, as has been mentioned, thought to have its cause on the decreased amount of serum protein, and so it is better preceived as manifestation of an automatic regulating function of part of protein metabolism exercised according to the varying amount of protein in serum.

In this respect it is thought that the fact that those having eosinophilia show no change of antitryptic index gives that this eosinophil white blood corpscule is related in some form or other with part of protein metabolism. From these points it is now possible to think antitryptic index broadens its scope to suggest by telling the inhibition degree against tryptase in tissue or serum.

3. *Formation of "tryptisches Ulkus" by Bordyreff.*[1]

We must not think the general fall of antitryptic index to be the cause for tryptic ulcers because most of ulcer patients show the normal value or a value little higher,

That the removal of affected parts by operations exerts no particular influence on the antitryptic index tells there does not exist any special relation directly between the parts and the index, which, moreover, makes us think it needless that antitryptic index points out only the inhibition degree for pancreas trypsin.

However, tryptic ulcers is formed in the locus where anemia has been caused because the serum inhibition disappears—it is possible to think thus with regard to the locus.

4. *Raab's opinion.*[3]

Raab ascribed the rare formation of cancers in the duodenum to the weak resistance of maligne tumour cells especially to the pancreas trypsin. The thought is possible that in gastric cancers the cells are not digested by trypsin, for the antitryptic index is big, and, therefore, among those whose antitryptic index is big and lasting there are many having a high degree of cachexia and showing ascites or peritonitis, but as the biopsy findings do not prove the relation between the degrees of metastasis and adhesion and the value on antitryptic index, antitryptic index does not serve to make comment upon Raab's opinions in the affirmative or in the negative.

5. *Differential diagnosis of gastric cancers and gastric ulcers.*

As is obvious from the experimental results, when antitryptic index is big there is generally a suspicion of a cancer and if the big index lasts long its formation is more certain. In this respect, when an ulcer case belonging to the non-changing class with the flower-bed-type in niche shows a big and lasting index, he should at any rate be warned that there is a symptom of ulcer changing into cancer. Again, in the changing class, the experimental results make us know that the niveau of changes is higher in cancers than in ulcers and that the position of the niveau is given by its mean value in each case. Furthermore, if we get the average of the lowest values of cancers, we should find it almost equal to the average of the whole values of ulcers. Similarly, the average of the highest values of ulcers approximates to the average of the whole values of cancers. These averages of the whole values of ulcers and of those of cancers can be regarded as the minimal value of cancers and the maximum value of ulcers respectively, which is clearly shown by the distribution conditions of the lowest values of cancers and the highest values of ulcers. Even when the values fall between these two averages, the differentiation between cancers and ulcers may be made by the directions their changes take (rising or falling). In other words we can pretty well differentiate one from the other by observing the magnitude of antitryptic index, especially its change by the course of time. In this case the values which are to be practically employed as a criterion of the dissemination are those average values given in Table VIII, the bordering values and the directions of changes in Tables IX and X.

Cancers and ulcers of changing and non-changing groups have, as I have stated, their clinical features peculiar to each. Here the character of changes is that ulcers which in ordinary conditions have a normal value sometimes run a rising course, i.e. showing the M-type change, while cancers which in most cases hold a high value sometimes fall near to the normal value, i.e. showing the W-type change, these two appearing to take opposite directions to each other. Accordingly the change of the former suggests there being an acute process, while that of the latter proves good prognosis (especially with respect to indication for operations). Thus the change of antitryptic index brought about in the process of time is qualified to judge of the nature of the pathological process, its degree of progress and prognosis of cancers and ulcers.

Summary and Conclusion.

As to the antitryptic index of gastric ulcer, duodenal ulcer and gastric cancer, the value itself does not bear any special relation directly to the pathological process, but is understood as a reaction against that of a living

thing and as an automatic regulating mechanism against a part of protein metabolism. According to nature, my " symplex-theory " is, I think, most satisfactorily fit to explain the above facts. Now by observing the change of antitryptic index, especially in the course of time, from the clinical point of view, we can see that the value of antitryptic index is very serviceable to make the differentiation of cancer from ulcer and when the two diseases are each classified under the two, changing and non-changing, classes each is found to have its distinct clinical features. From these respects the change of antitryptic index may be employed as a mean to know, to some extent, the nature of the pathological process and its degree of process and to judge the prognosis.

The above statement may be briefly summarised as follows:

1. Among ulcer patients very few show the change of antitryptic index who have eosinophilia.

2. As to gastric ulcer patients, many have the tent-type or finger-trace-type of niche and acute process among those having a big highest value of antitryptic index and those belonging to the changing group, while there are many with flower-bed-type of niche and indicating chronic process among those who have a small highest value of antitryptic index and those falling under the class of changing.

3. In respect to duodenal ulcers, many in unfavorable progress are found in the changing class, and many in favorable progress are found in the non-changing class.

4. In respect to gastric cancers many presenting ascites or peritonitis and having high degree of cathexia are found among those with a big lowest value and those of the non-changing class, while only few belong to the changing group and have a small lowest value.

5. Antitryptic index of gastric cancer is generally big in comparison with that of ulcer and more patients of cancer show a big value than those of ulcer. The average value in the non-changing class, the average, highest and lowest values in the changing class are respectively bigger with cancer patients than with ulcer, and those who values are big are also more in cancer cases. In the changing class, the minimal value of cancers and the maximal of ulcers are respectively given by the averages of the whole values of ulcers and of those of cancers. The directions of changes are naturally opposite to each other.

References.

1) Bordyreff, Ergeb. inn. Med. u. Kinderhk., 1938, **54**, 333.

2) Stuber, Ztschr. exp. Path. u. Ther., 1914, **16**, 295.

3) Raab and Breuer, Ztschr. ges. exp. Med., 1935, **96**, 603; **97**, 413; Raab, Klin. Wschr., 1935, **14**, 1633.

4) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 269.
5) Chrometzka, Ztschr. ges. exp. Med., 1930, **69**, 656, 665; 1932, **80**, 395, 408, 420.
6) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 283.

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Action of Serum upon Proteolysis.

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In the previous paper[1] concerning antitrypsin of serum I stated that the rise and fall of it should, according to its nature, to be understood as a manifestation of an automatical regulating mechanism for the protein metabolism in the livings. About "antipepsin", today, many workers are in denial. As to cathepsin action, however, Kosaki[2] stated that it was inhibited in the presence of serum, which was opposite to the results of Purr's[3] experiments, who showed that it underwent some activation in the presence of serum. On the other hand, about ereptase action Matsuo[4] recently demonstrated that the hydrolysis of pepton and diglycin by intestinal juice of a dog were indifferent from the action of serum. In view of these respects I intend to attain in the present paper to a further knowledge of the action of serum upon the proteolytic enzymes which ought to play an important part in protein metabolism in the livings.

Experimental.

Method and Materials.

Enzyme: organ-maceration 1+glycerin-water (1:1) 3; human gastric mucosa from biopsy; Substrate: resolved in buffer (m/15-phosphate- or m/10-citrate-n/10-HCl-buffer); Plasma: 3.8%-citrate-plasma (4:1).

Increased acidity in 4 c.c. digestate formol-titrated with 0.1 n-NaOH, after the digestion at 37°C under toluol; For control, experiments without substrate or enzyme with and without serum on one hand, and only with serum without substarate and enzyme on the other, under the same condition carried out. Figures of tables show corrected values.

Results.

1. *Proteolysis of rabbit gastric mucosa.*

The hydrolysis of casein at pH 4.5–5.0 is in the presence of human serum or plasma apparently activated, but that of pepton remains un-

affected. With red blood corpsucle on activation upon casein hydrolysis was found contrary to the results observed by Purr.[3] Moreover, also hydrolysis of casein and serum albumin at pH 1.9 undergo no influence.

Table I.

Action of Human Serum upon Proteolysis of Gastric Mucosa (Rabbit) (1).

2 c.c. maceration juice+20 c.c. substrate buffer solution+0.5~100 c.c. serum or buffer.

Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH

% Substrate 20 c.c.	Time in hrs. / pH	without serum		with 0.5 c.c. serum		with 1 c.c. serum		with 0.5** c.c. plasma	
		4.5	5.0	4.5	5.0	4.5	5.0	4.5	5.0
0.5% Casein (Hammarsten)	24	0.25	0.15	0.35	0.25	0.35	0.25	0.30	0.25*
	72	0.35	0.25	0.5	0.4	0.5	0.4	0.45	0.25*
	120	0.5	0.35	0.75	0.5	0.75	0.5	0.75	0.35*
1% Pepton (Kyokuto)	24	0.25	0.1	0.35	0.1	—	0.1	—	0.2*
	72	0.4	0.15	0.45	0.2	—	0.15	—	0.2*
	120	0.6	0.25	0.6	0.3	—	0.25	—	0.3*
Control without substrate	24	0.05	0	0	0	0	0	0	0
	72	0.05	0	0	0	0	0	0	0.05
	120	0.05	0	0.05	0	0	0.05	0	−0.05

Corrected values are given. * with 0.1 c.c. red blood corpuscles suspension, ** 3.8% citrate plasma (4: 1).

Table II.

Action of Human Serum upon Proteolysis of Gastric Mucosa (Rabbit) (2).

2 c.c. maceration juice+20 c.c. substrate buffer solution+0.5–1.0 c.c. serum of buffer; pH: 1.9–2.2.

Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH.

% Substrate 20 c.c.	Time in hrs.	without serum	with 0.5 c.c. serum	with 1 c.c. serum
0.5% Casein (Hammarsten)	24	0.2	0.2	0.2
	72	0.2	0.2	0.2
	120	0.35	0.3	0.35
1% Serumalbumin (E. Merck)	24	0.35	—	0.3
	72	0.45	—	0.45
Control without substrate	24	0	0	0.05
	72	0.05	0	0.05
	120	0.05	0	0.05

Corrected values are given.

2. *Proteolysis of rabbit liver.*

Contrary to the above results the hydrolysis of casein at pH 4.5 is inhibited in the presence of serum, while that of pepton by maceration which underwent acid treatment (pH 4.0, 40 min., 37°C) to be free from ereptase on one hand, and also that of pepton at pH 7.8 by the maceration which was treated with alkaline (pH 9.5, 2 hrs., 37°C) and was made free from catheptase on the other hand, these remain unaffected.

TABLE III.

Action of Human Serum upon Proteolysis of Liver (Rabbit).

2 c.c. maceration juice+18 c.c. substrate-buffer solution+1 c.c. serum or buffer.

		Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH	
% Substrate, 18 c.c. pH, & enzyme*	Time in hrs.	without serum	with 1 c.c. serum
0.5% Casein (Hammarsten), pH; 4.5; enzyme (a)*	24	0.2	0.05
	48	0.2	0.1
	120	0.25	0.1
0.5% Pepton (Kyokuto); pH: 4.5; enzyme (a)*	24	0.1	0.05
	48	0.1	0.1
	120	0.2	0.15
the same as above; enzyme (b)*	24	0	—
	48	0.05	—
	120	0.05	—
the same as above; pH: 7.8; enzyme (a)*	24	0	—
	48	0	—
	120	0.05	—
the same as above; pH; 7.8 enzyme (b)*	24	0.1	0.15
	48	0.15	0.15
	120	0.65	0.6

* (a), free from ereptase activity, (b), free from catheptic peptonase activity after the acid or alkaline treatment by the method of Utzino and Sakai.

3. *Proteolysis of human gastric mucosa.*

The hydrolyses of casein and serum albumin at pH 4.4–5.0 undergo no influence or are rather inhibited in the presence of serum, while that of egg albumin is apparently activated to such an extent as is achieved with cystein activation. On the contrary, pepton hydrolysis at pH 4.6–4.2 and casein hydrolysis at pH 2.0–2.5 respectively undergo no influence.

Discussion.

The fact that the action of catheptic pepton splitting enzyme (cathopeptonase[5]) which may be widely distributed in animal tissues is indifferent

TABLE IV.

Action of Serum upon Proteolysis of Human Gastric Mucosa.

Gastric mucosa obtained from biopsy (cancer); 2 c.c. maceration juice+18 (20 c.c.) c.c. substrate-buffer solution+0.5–2 c.c. serum or buffer; pH 2.0–5.0.

Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH

% Substrate c.c.	pH	Time in hrs.	without serum	c.c. serum added		
				0.5	1	2
1% Casein (Hammarsten) 18 c c.	2.0	24	0.1	0.1	0.1	0.1
		48	0.1	0.1	0.15	0.2
		72	0.2	0.2	0.2	0.2
	2.5	24	0.1	—	0.1	—
		48	0.1	—	0.15	—
		72	0.2	—	0.2	—
	4.4	24	0.15	0.15	0.05	—
		48	0.3	0.25	0.2	—
		72	0.3	0.4	0.25	—
	5.0	24	0.1	0.05	0	—
		48	0.1	0	0.05	—
		72	0.1	0.1	0.1	
1% Serum albumin (E. Merck) 18 c.c.	5.0	24	0.3	—	0.15	—
		48	0.3	—	0.15	—
		72	0.35	—	0.2	—
0.5% Ovalbumin (E. Merck) 20 c.c.	4.6	24	0.1	—	0.15	—
		48	0.1	—	0.25	—
		72	0.15	—	0.3	—
1% Pepton (Kyokuto) 2 c.c.	4.2	24	0.2	—	0.15	—
		48	0.2	—	0.25	
		72	0.2	—	0.25	
	4.6	24	0.25	—	0.2	
		48	0.4	—	0.25	
		72	0.4	—	0.4	

from that of serum might be concluded, together with those of Matsuo[4] about ereptase activity, that peptidase activity in general is by no means affected by serum action. Moreover a serum "anticathepsin", which was referred to by Kosaki,[2] can not hold itself definitely, for the action of serum upon catheptic enzyme is varying with the sort of animal, organ and substrate employed. This is really the case also when an activating ability of serum for cathepsin observed by Purr[3] is taken into account. Although in some cases catheptic hydrolysis undergoes activation in the presence of the serum, we can not attribute it simply to an action of some SH-compound in serum only, because, though SH-compound should activates both of the catheptic hydrolysis of protein and pepton, the above

experiments showed only the activation of the catheptic hydrolysis of protein (casein).

On the other hand real existence of so called "antipepsin" is from the above results very questionable, for protein hydrolysis at pH 2.0–2.5 remained always unaffected with serum present.

Summary.

1. The hydrolysis of casein at pH 4.5–5.0 by gastric mucosa of a rabbit is activated apparently in the presence of serum or plasma, while that of pepton and also that of casein or serum albumin at pH near 2.0 always remain unaffected.

2. The hydrolysis of casein at pH 4.5 by rabbit liver is inhibited by serum but that of pepton at pH 4.5 or 7.8 is not. These two pepton splitting activities can be separated from each other by alkaline or acid treatment.

3. The hydrolysis of casein and serum albumin at pH 4.4–5.0 by gastric mucosa (human) are to some extent inhibited by serum or scarecely undergo influence, while that of egg albumin is activated by serum. The hydrolysis of pepton at pH 4.6 and that of casein or serum albumin at pH 2.0, all remain without being influenced.

4. Catheptic pepton splitting enzyme action (cathopeptonase) may presumably be in a rabbit's liver, in its gastric mucosa and also in human gastric mucosa.

References.

1) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 291.
2) Kosaki and Ishikawa, Jap. Journ. Gastroentr., 1939, **11**, 101.
3) Purr and Russel, Ztschr. physiol. Chem., 1934, **228**, 194.
4) Matsuo, Tohoku Journ. Exp. Med., 1941, **41**, 108.
5) Sakai, Journ. Biochem., 1944, **36**, 443.

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An Enzymological Study for Genesis of Gastric Ulcer.

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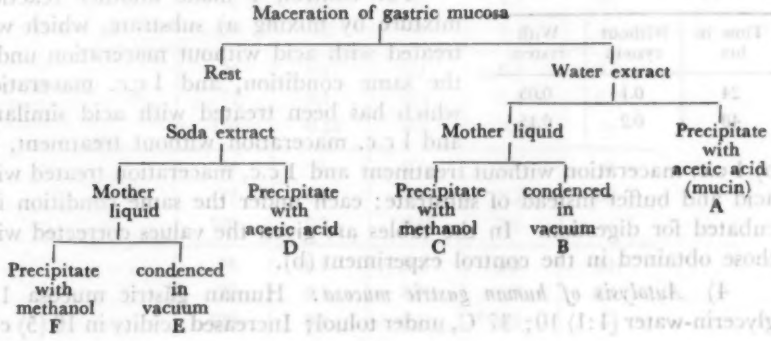
Introduction.

If we consider the cause of gastric ulcer according to the leading theory of E. v. Bergmann (1936) to be attributed to local anemia owing to spasm of small vessels, the splitting activity of the catheptase in the locus should be activated. Consequently, it is very interesting question whether or no the mucosa protein, which, Asao[1] showed, has resistency against pepsin action, will lose this resistency against the pepsin action following even after the cathepsin action for so short a time that no increase in amino-N may be observed. If an affirmative answer for the question might be expected, what was mentioned above would be a fact. This is of importance for the genesis of gastric ulcer when the matter is dealt with enzymologically.

Experimental.

Methods and Materials.

Following protein fractions (A-F) obtained by Hirooka[2] from gastric mucosa of swine were used as substrate.



Amount of N[2] and ash,[2] and sulphur reaction of these fractions (A-F) are given in Table I.

TABLE I.
Protein Fraction of Gastric Mucosa (Swine).

Fraction	A	B	C	D	E	F
N-content[2], %	12.9	12.7	13.3	13.2	12.1	11.7
Ash,[2] %	2.8	3.1	0	3.4	0.65	2.5
Sulphur reaction	−	++	++	−	+	±

Method: 1) *Acid treatment for the substrate:* Constant amount of substrate was resolved in n/10-NaOH or HCl and its pH value was made adjusted for the experiments, then 1 c.c. maceration juice (gastric mucosa 1: glycerin-water (1:1) 3, pH 4.0–5.0, rabbit) was added and incubated at 37°C.

TABLE II.
Proteolytic Activity of Gastric Mucosa (Rabbit) after the Alkaline Treatment.
pH; 4.5

15 c.c. 0.5 % casein (Hammarsten)-buffer solution + 3 c.c. maceration juice; alkaline treatment: pH 7.7, 37°C, 40 min.; cystein activation: 2 c.c. enzyme + 0.024 grm. cystein-HCl in 1 c.c. H_2O, neutralized with NaOH, 37°C, 30 min. Increased acidity in 4 c.c. digestate was formol-titrated with 0.1 n-NaOH; c.c.

Time in hrs.	Without cystein	With cystein
24	0.1	0.05
48	0.2	0.15

2) *Removing the catheptase activity:* The maceration juice after the alkaline treatment by following the method of Utzino and Sakai[3] (pH 7.7, 40 min., 37°C) becomes free from cathepsin action (Table II).

3) *Reaction system:* After the acid treatment I added first n/10-HCl to correcting its pH value to 2.0, next citrate-buffer (m/10, pH 2.0) making its concentration 1–2% and then 1 c.c. enzyme (pepsin). This reaction mixture was incubated for digestion. Increased acidity in 4 c.c. digestate was formol-titrated (0.1 n-NaOH).

For control, I made another reaction mixture by mixing a) substrate, which was treated with acid without maceration under the same condition, and 1 c.c. maceration which has been treated with acid similarly and 1 c.c. maceration without treatment, or b) 1 c.c. maceration without treatment and 1 c.c. maceration treated with acid and buffer instead of substrate: each under the same condition incubated for digestion. In the tables are given the values corrected with those obtained in the control experiment (b).

4) *Autolysis of human gastric mucosa:* Human gastric mucosa 1+ glycerin-water (1:1) 10; 37°C, under toluol; Increased acidity in 10 (5) c.c.

digestate was measured by formol-titration; gastric mucosa obtained from biopsy (ulcer, 54 years, male).

Results.

I. The Action of Pepsin upon the Protein of Gastric Mucosa (Swine).

No difference between the degrees of peptic hydrolysis of these protein fractions is found when peptic activity is relatively strong (maceration 1:3). On the contrary, when diluted maceration juice (1:30) is employed, of which peptic acitvity is though weaker than before, the degrees of hydrolysis of fractions B, C, E and F are much smaller than those of A and D, which indicates that fractions B, C, E and F are more resistent than the other (Table III).

TABLE III.

Peptic Digestibility of Mucosa Protein (Swine).

10 c.c. 2 (1) %-substrate-buffer solution + 2 c.c. maceration juice (gastric juice)* + 10 c.c. m/10-citrate-n/10-HCl-buffer. pH 1.9–2.2. * Rabbit gastric mucosa.

Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH					
Protein fraction	Time in hrs.	Gastric juice (human) 1:1	Maceration juice of gastric mucosa 1:10	1:3	1:30
A	24	0.2	0.1	0.25	0.15
	72	0.2	0.2	0.25	0.2
	120	0.25	0.3	3.0	—
B	24	0.05	0.1	0	0
	72	0.1	0.2	0.2	0.1
	120	0.15	0.25	0.2	—
C	24	0.1	0	0.2	0
	72	0.2	0	0.3	0.1
	120	0.2	0	0.35	—
D	24	0.1	0.1	0.2	0.3
	72	0.1	0.1	0.4	0.35
	120	0.15	0.2	0.45	—
E	24	0	0.1	0.2	0
	72	0.2	0.1	0.25	0.1
	120	0.2	0.15	0.3	—
F	24	0.1	0.1	0.2	0.1
	72	0.1	0.1	0.3	0.1
	120	0.1	0.1	0.3	—

Then in the following, because of the stronger resistency of fraction B or C the experiments were attempted only with them to examine the change of their resistency against peptic digestion, caused by the acid treatment. Distinct increase in the degree of the hydrolysis of fraction B or C can be observed after the acid treatment for the substrate, when instead of pepsin with strong activity diluted one is employed (Tables IV–VI).

TABLE IV.

Peptic Digestibility of Fraction B Before and After the Acid Treatment (1).

(10 c.c. 0.5% substrate+1 c.c. maceration juice)+1 c.c. enzyme (pepsin)+12 c.c. m/10-citrate-n/10-HCl-buffer; ( ): acid treatment at pH 4.5; 37°C; digestion: pH 2.2., 37°C.

Enzyme	Time in hrs.	without acid treatment	Time in hrs. of acid treatment			
			½	1	2	3
		Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH				
Gastric mucosa (1:24), rabbit	24	0.05	0.15	0.15	0.15	—
	48	0.1	0.2	0.2	0.25	
Gastric juice (1:10), human	24	−0.05	—	0.1	0.1	0.15
	72	0.1	—	0.65	0.6	0.75

TABLE V.

Peptic Digestibility of Fraction B Before and After the Acid Treatment (2).

(10 c.c. 0.5% substrate+1 c.c. maceration juice)+1 c.c. pepsin+11 c.c. buffer; ( ): acid treatment: pH 4.9, 1 hr., 37°C; Pepsin: rabitt gastric mucosa; digestion: pH 2.0 37°C.

Enzyme (dil.)	Time in hrs.	Without acid treatment	Acid treatment with maceration free from cathepsin activity	With acid treatment, as usual
		Increased acidity in 4 c.c. digestate; c.c. 0.1 n-NaOH		
1:3	24	0.1	—	0.1
	72	0.15	—	0.2
	120	0.2	—	0.2
1:12	24	0.05	0	0.15
	72	0.1	0.1	0.2
	120	0.1	0.1	0.3

TABLE VI.

Peptic Digestibility of Fraction C Before and After the Acid Treatment.

(10 c.c. 1% substrate 1 c.c. maceration juice)+1 c.c. pepsin+10 c.c. m/10-citrate-n/10-HCl-buffer; ( ): acid treatment for substrate at pH 4.5; pepsin: rabbit's gastric mucosa; digestion: pH 2.0, 37°C.

		Increased acidity in 4 c.c. digestate; c.c., 0.1 n-NaOH				
Pepsin (dil.)	Time in hrs.	With 30 min. treatment	With 1 hr. treatment	Without treatment	Treated without maceration juice	Treated with maceration, free from cathepsin action
1:3	24	—	0.45	0.35	—	—
	48	—	0.6	0.6	—	—
1:24	24	0.25	0.4	0.1	0.25	0.2
	48	0.3	0.65	0.25	0.3	0.25

In each case, however, the changing degree of the resistency is independent of the duration of the treatment. And naturally no increase in acidity under these reaction systems is observed after such treatment with acid only (Table VII). And further it is not misleading to conclude that the above results are not simply due to the action of an acid, because in the control experiment the substrate is also treated similarly with acid, without maceration juice. Moreover, pH value employed for the acid treatment is nearly the same with the optimal pH value of catheptic action. In order to confirm that the change of resistency results from the cathepsin action in maceration juice, further experiments were carried out, in which the substrate was treated similarly with maceration which had beforehand been treated with alkaline and made free from cathepsin action. No difference in the degree of hydrolysis before and after the acid treatment with such maceration is found (Tables V–VI).

TABLE VII.

Acidity Increased After the Acid Treatment for Substrate Only.

20 c.c. 1% substrate-buffer solution +1 c.c. maceration juice (1:3); pH 4.5; 37°C; Increased acidity in 4 c.c. digestate; formol titration; 0.1 n-NaOH, c.c.

Fraction	Time in hrs.	Increased acidity
B	1	0
	2	0
C	1	−0.05
	2.	−0.05

By the by, the degree of hydrolysis by pepsin with weaker activity of the fraction B or C, which has been made less resistent, is nearly similar to those by pepsin of strong activity of these fractions which underwent no acid treatment (Tables IV–VI). This is of interest when we consider the hydrolysing mechanism of the protein molecule.

II. Autolysis of Human Gastric Mucosa.

The optimal pH value for autolysis of human gastric mucosa in acid side lies in pH 4.5 and in alkaline side in 8.0 corresponding to those of cathepsin and tryptase action respectively, but not to that of pepton.

TABLE VIII-a.

Autolysis of Human Gastric Mucosa.
(1) Experiments in acid side.

Increased acidity in 10 c.c. digestate; c.c. 0.1 n-NaOH						
Time in hrs.	pH					
	2.0	3.0	4.0	4.5	5.0	6.2
48	0.5	0.6	0.8	1.2	0.6	0.5
96	0.55	0.65	0.85	1.45	1.0	0.6

TABLE VIII-b.

Autolysis of Human Gastric Mucosa.
(2) Experiments in alkaline side.

Increased acidity in 5 c.c. digestate; 0.1 n-NaOH				
Time in hrs.	pH			
	7.2	7.5	8.0	8.5
48	0.1	0.15	0.2	0.1
96	0.15	0.15	0.25	0.15
150	0.15	0.2	0.35	0.25

DISCUSSION.

From the results viewd above it is evident that gastric mucosa protein itself is usually resistent against pepsin action. We may conclude that it is attributed to a result of the cathepsin action in the gastric mucosa that this resistency apparently decrease after the acid treatment.

Concerning the change of resistency, universally, of protein against the protease, Abderhalden[4] demonstrated that serum globulin came to easily cleave by the following pancreas trypsin action after the action of pepsin-HCl (48 hrs.) in which no increase in acidity had been observed. Recently Aratani[5] showed an apparent activation of peptide binding in the egg albumin and serum albumin as a result of pepsin action (pH 4.4, 5–20 min. 37°C) in which no increase in acidity was observed. Although he presumed at that time the existence of an enzyme " denaturase ", many workers are of an opinion on the point that the degenerated albumin will lose its resistency against pancreas trypsin. Also in the Neurath's[6] ex-

periments, it was shown that the degree of hydrolysis of serum albumin (horse) regenerated from guanidin-HCl, by pancreatin (u.s, p. (x) Merck) is still larger than that of the native one.

Analogous to these facts, the change of the resistency of protein of gastric mucosa against pepsin action as observed above may, according to the opinion of Lindenström-Lang,[7] be attributed to the degeneration of the mucosa protein, as a result of the first stage of hydrolysis by cathepsin action. Furthermore, not incompatibly with the fact that the mucosa protein, as is obviously known from the experiment on autolysis, is easily cleft by cathepsin, it may be concluded that the cathepsin in gastric mucosa should play the first important part of the gastric ulcer. In this respect it is suggestive that sulphur reactions of fractions B and C with which my preceding experiments were tried, were both strong positive (Table I). Recently Harry H. Le Veen[8] showed that ulcer which had been caused by ligation of all vessels to the stomach of dogs, grew unusually in larger curvature. Although in this case these ulcers may be attributed to the cathepsin action, this is not universally applicable for ulcer genesis, because many ulcers which are observed clinically show always some factors acting on the surface of mucosa. Those factors have been commonly thought to be the action of pepsin-HCl. And Philip and Price,[9] who made his experiments on dogs, showed also that even connective tissues were easily digested by hyperacid gastric juice histamin produced. From the result of my experiments, however, it seems difficult to understand that only pepsin-HCl is to be responsible for the cause of the ulcer.

Now the facts that the optimal pH value for autolysis of gastric mucosa in alkaline side lies in near 8.0 and that gastric mucosa itself has also the tryptic activity,[10] these indicate the possibility of tryptic ulcer genesis besides the peptic one.

Then it is conclusively possible to make an enzymological assupmtion about ulcer genesis, indorsing Bergmann's theory, as follows: pH value there may be in weak acid side owing to the anemia of the locus and the cathepsin there should be activated to hydrolyzing the protein with which the mucosa of the locus is constructed, and secondarily the mucosa protein there will lose its resistency against pepsin or trypsin action, being degenerated by cathepsin action, which may result in growth of ulcer in the locus, where the mucosa should be acted gradually be pepsin or trypsin from the surface. In such a case 30 min. or 1 hr. of the contraction of small vessels resulting in local anemia may be enough for ulcer genesis.

Summary and Conclusion.

1. Gastric mucosa protein (swine) itself is usually reistent against pepsin action with exception of so-called mucin.

2. This protein loses its resistency against pepsin after the acid treatment with maceration juice of gastric mucosa (pH 4.5–4.9, 30 min.–1 hr., 37°C).

3. Acid treatment with maceration which has been beforehand made free from its cathepsin action by alkaline treatment (pH 7.7, 40 min., 37°C) gives no influence upon resistency of this protein.

4. The optimal pH values for autolysis of human gastric mucosa are found in 4.5 in acid side and in 8.0 in alkaline side. These pH values correspond to those of cathepsin and trypsin action respectively, but not to that of pepsin.

5. The first stage of enzymological mechanism of the ulcer genesis may be degeneration of the mucosa protein of the locus which is anemic and anaerob owing to the contraction of small vessels. This degeneration may be caused catheptic enzyme action in the locus. Secondarily the protein there loses its resistency against pepsin or trypsin action, which results in ulcer by the action of pepsin or trypsin from the mucosa surface.

References.

1) Asao, Journ. Biochem., 1932, **16**, 17.
2) Hirooka, Tohoku Journ. Exp. Med., 1942, **42**, 309.
3) Utzino and Sakai, Journ. Biochem., 1941, **33**, 457.
4) Abderhalden, Fermentforsch., 1928, **10**, 12.
5) Aratani, Journ. Jap. Biochem. Soc., 1949, **21**, 1.
6) Neurath, Journ. Gen. Physiol., 1943, **26**, 513.
7) Lindenström-Lang, Proc. Roy. Soc. (London), 1939, B **127**, 17.
8) Le Veen, Surg. Gyn. Obstr., 1948, **86**, 164.
9) Philip and Price, Surg. Gyn. Obstr., 1947, **84**, 959.
10) Hayakawa, Tohoku Journ. Exp. Med., this volume, p. 303.

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Studies on the Cardiac Rhythm of the Dog in Experimental Hyper- and Hypo-thyroidism.

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Introduction.

It is well known that the tachycardia is one of the most conspicuous signs of the hyperthyroidism of man. Boas and Weiss[1] who studied the features of the cardiac rhythm of patients in hyperthyroid states by means of Boas' "*Cardiotachometer*", a device to record the changes in cardiac rate continuously, noticed that the tachycardias characteristic to these patients persisted even in the hour of sleep at night.

By this method of study, however, as by the ordinary method of counting the pulse rate for a certain interval of time, no information can be obtained about the instantaneous changes in cardiac rate which, indeed, seem to be of interest from the physiological standpoint. Recently Taguchi[2] noticed, after having studied many cases of hyperthyroidism by the use of Matsuda's "*Cardiotachograph*"[3] which records automatically the length of each pulse interval as ordinate to the accuracy of one hundredth of a second, an interesting fact that in every case of hyperthyroidism the respiratory variations of cardiac rhythm vanished almost completely, but reappeared gradually along with the improvement of general symptoms by the institution of treatments such as Lugol's solution by mouth followed by the subtotal thyroidectomy.

To reinvestigate his results by animal experiments, I studied the changes in cardiac rhythm of dogs by means of cardiotachograph in states of hyperthyroidism by feeding of desiccated thyroid glands as well as of hypothyroidism following thyroidectomy. And as I got, in addition to the confirmation of Taguchi's conclusions, some interesting results, I will report them in the following pages.

Experimental.

Methods.

The experiments consisted of the observations of pulse rhythms of dogs in two conditions, viz.: at first in a state of hyperthyroidism and then of hypothyroidism, both in the same animal. Five healthy dogs were used, four for the experiments and one as a control. The hyperthyroidism was brought about by feeding desiccated thyroid glands (prepared by Fujisawa's Co., according to the Japanese Pharmacopeia), 0.3 g per Kg of body weight per day continuously. About a week after the start of the medication there appeared various signs characteristic to hyperthyroidism, such as emaciation, acceleration of heart, diarrhea, thirst and polyphagia, at times perverted appetite and restlessness. These signs got aggravated rapidly until, in the twenty fourth day of the experiment, one dog died under the picture of severe thyrotoxicosis with generalized convulsions. Slight exophthalmus and turbidity of the cornea was observed in one case.

At this stage the thyroid feeding was discontinued; thereafter, all the dogs which survived recovered rapidly from the diseased states.

After they regained good health and their body weights attained the initial levels (over thirty days being necessary for it), an operation of bilateral thyroidectomy was performed on each with especial care not to damage the parathyroid glands. Healing of wounds was satisfactory, contrary to Palmer's opinion[4] that thyroidectomy causes a lowering of animal's resistance to infection and a difficulty of wound healing. Following the operation the animals became rather sluggish and body weights decreased to a moderate extent. While no definite sign of general puffiness was observed, hairs lost its luster and fell off more and more. The three dogs died of general weakness, thirty eight, fifty six days and seven months after the operation respectively.

Automatic registration of cardiac rate was performed by Matsuda's cardiotachograph.[3] Its principle may be described here briefly. A discharge was effected in a thyratron bulb by an amplified action potential of dog's heart (the deflection R in ECG). Because of a condenser put parallel to the bulb, the interval between two successive cardiac systoles determines the potential gradient along the plate resistance at the moment of each discharge. When this potential gradient, after a suitable amplification, is recorded by an oscillograph, the length of each spike will correspond to each pulse interval (Fig. 1).

The cardiac action potential was lead from two needle electrodes inserted subcutaneously in the right shoulder and left thigh.

Respirations were recorded by an electrical pneumograph.

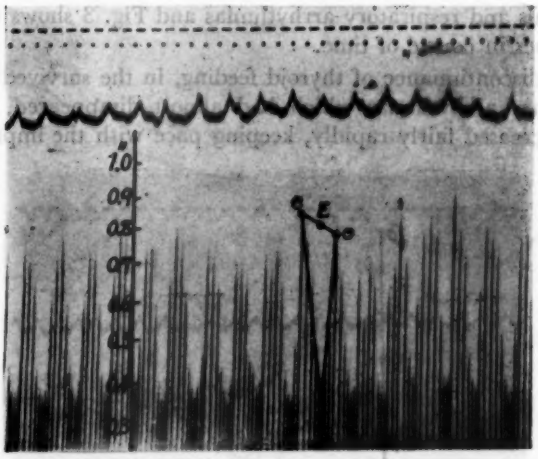

Fig. 1. Cardiotachogram of dog (No. 1).
Normal condition.
Upper: Time mark, in two second interval.
Middle: Respiration, inspiration upward.
Lower: Cardiotachogram.
Each vertical line corresponds to a pulse interval, its value to be read by the scale on left.
Triangle in the middle part indicates evaluation of respiratory arrhythmia. E (average of two *e* s) and I indicate expiratory and inspiratory pulse intervals respectively, the difference of both values is the extent of respiratory arrhythmia.

Results.

It is a well known fact that dogs have marked respiratory arrhythmias. As a quantitative measure of this type of arrhythmias the difference between the pulse intervals in the inspiratory and the expiratory phases of respiration was used (Fig. 1). The extent of the respiratory arrhythmias thus measured of five normal dogs under conditions of rest were 0.30~0.47 (0.39) sec., 0.45~0.71 (0.62) sec., 0.24~0.68 (0.50) sec., 0.27~0.40 (0.34) sec. and 0.52~0.74 (0.66) sec. respectively, the values in parenthesis being the averages in each individual dog. In the control dog the respiratory arrhythmias remained essentially unchanged throughout the period of experiment.

All the four dogs upon which the thyroid feeding was instituted, showed distinct changes in the features of cardiac rhythm. Along with the development of the tachycardias and various thyrotoxic manifestations stated above, the respiratory arrhythmias decreased day by day (Fig. 2) until, after about two weeks, the cardiotachogram exhibited a picture of so called "Puls-starre" (rigid pulse). Table I is the data of the average

pulse intervals and respiratory arrhythmias and Fig. 3 shows the changes of these values in course of time.

By the discontinuance of thyroid feeding, in the survived three dogs, the respiratory arrhythmias which had almost disappeared, reappeared soon. It increased fairly rapidly, keeping pace with the improvement of

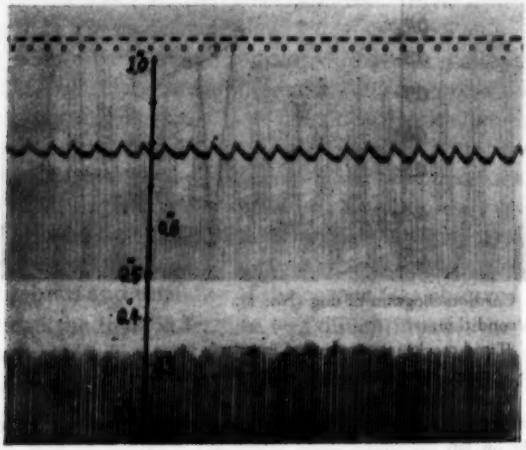

Fig. 2. Cardiotachogram of dog (No. 1) in experimental hyperthyrodism. Note markedly decreased respiratory arrhythmia.

TABLE I.

Pulse Intervals and Respiratory Arrhythmias (in Seconds) of Dogs in Experimental Hyperthyroidism.

Dogs		Before feeding	Days of feeding							
			5	7	9	12	13	14	17	18
No. 1	P. I.	0.80	0.72			0.49		0.36		
	R.A.	0.39	0.41			0.15		0.09		
No. 2	P. I.	1.06	0.71	0.33	0.36					
	R.A.	0.62	0.25	0.03	0.03					
No. 3	P. I.	1.01	0.57				0.62		0.31	Death
	R.A.	0.50	0.23				0.22		0.02	
No. 4	P. I.	0.73		0.56		0.35				
	R.A.	0.34		0.16		0.06				

P.I.=Average pulse interval, that is the value obtained by dividing 60 seconds by the average pulse rate per minute.

R.A.=Respiratory arrhythmia measured by the differences between the pulse intervals in inspiratory and expiratory phases. Average value of over ten respiratory cycles.

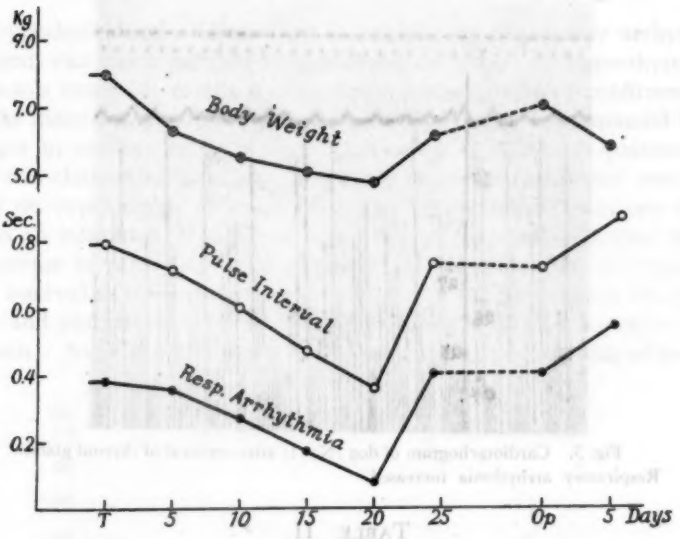

Fig. 3. Changes in body weight, pulse interval and respiratory arrhythmia of dog (No. 1) in experimental hyper- and hypo-thyroidism.
T: Start of thyroid feeding.
Op: Thyroidectomy.

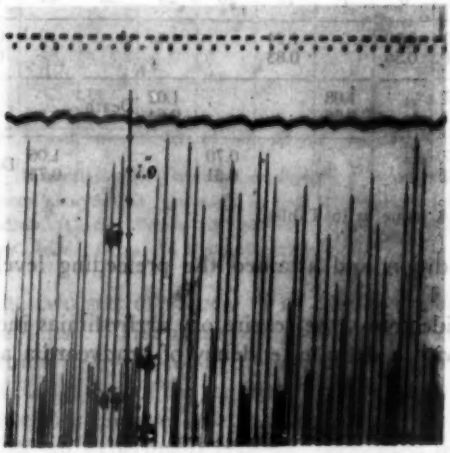

Fig. 4. Cardiotachogram of dog (No. 1) after recovery from hyperthyroidism. Reappearance of respiratory arrhythmia. Compare with Fig. 1.

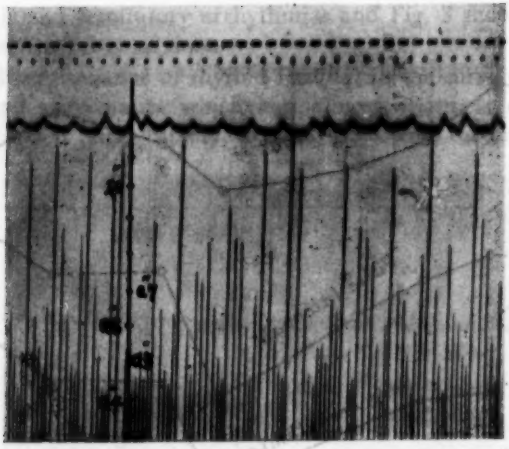

Fig. 5. Cardiotachogram of dog (No. 1) after removal of thyroid glands. Respiratory arrhythmia increased.

TABLE II.

Pulse Intervals and Respiratory Arrhythmias (in Seconds) of Dogs After Thyroidectomy.

Dogs		Before operation	Days after operation									
			6	7	17	20	25	38	43	56	86	210
No. 1	P. I.	0.70	0.90		1.24					0.94	0.83	Death
	R.A.	0.41	0.56		0.83					0.64	0.45	
No. 2	P. I.	0.67		1.08			1.02	Death				
	R.A.	0.21		0.66			0.64					
No. 3	P. I.	0.57				0.70			1.06	Death		
	R.A.	0.22				0.31			0.73			

P.I. and R.A. same as in Table I.

the general condition and attained the prefeeding level within a week (Fig. 3 and Fig. 4).

After thyroidectomy, the respiratory arrhythmias increased gradually (Fig. 5) along with a definite tendency of bradycardia as summarized in Table II.

DISCUSSION.

The results of the present experiments in dogs show clearly that the respiratory arrhythmias decrease in the hyperthyroidism and increase in

the hypothyroidism. The course in which the respiratory arrhythmias changed, ran quite parallel to the degree of hyper- or hypo-thyroidism. Taguchi's results as to the human hyperthyroidism were confirmed.

As these changes in respiratory arrhythmia were accompanied by the changes in cardiac rate, it seems interesting to compare quantitatively these two changes each other. According to Anrep and others' conclusion based on experiments in dogs,[5] the respiratory arrhythmias are chiefly due to an inhibition of efferent vagal tone on inspiration, rather than to an increase in vagal tone on expiration. I, therefore, took the expiratory pulse interval as the standard value not influenced particularly by respiration; and plotted the respiratory arrhythmias against the expiratory pulse intervals. As evidenced clearly in Fig. 6, a distinct relationship of lineality

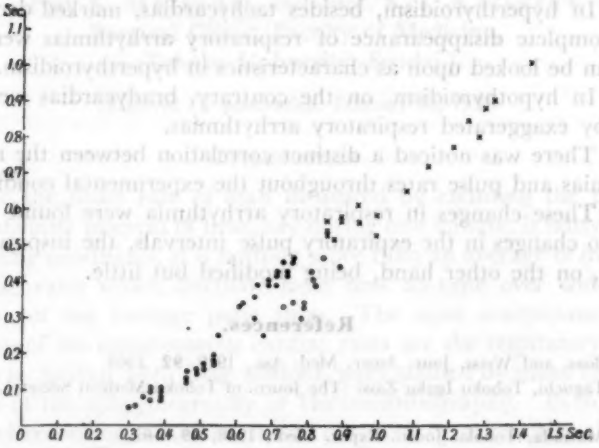

Fig. 6. Interrelationship between expiratory pulse intervals (abscissa) and respiratory arrhythmias (ordinate) in dogs in experimental hyper- and hypo-thyroidism. Circle: Before experiments. Point: In hyperthyroidism and recovery phase. Cross: In hypothyroidism.

exists between the two sets of values. The line of trend makes an angle of 45° with the abscissa. This means that the decrease or increase in respiratory arrhythmia in hyper- or hypo-thyroidism are brought about by a shortening or an elongation of the expiratory pulse intervals while the inspiratory pulse intervals change little. This findings are quite in accord with the results of an experiment recently performed in our laboratory concerning the effects of atropin or other drugs upon the respiratory arrhythmias in dogs.

Also in man, Matsuda and Suzuki[6] and I confirmed the existence

of a similar relation of distinct lineality between the respiratory arrhythmias and expiratory pulse intervals in states of physiological activities. Details of these studies will be reported in this Journal.

While the physiological mechanism of the tachycardias in hyperthyroidism is not discussed here as it is not a main subject in this report, it is firmly established that the pulse rate itself is one of the most powerful factors to determine the extent of respiratory arrhythmias, no matter what the cause of tachycardia or bradycardia may be.

Summary.

1) The cardiac rhythms of dogs, the respiratory arrhythmias in particular, were studied by "*Cardiotachograph*" in experimentally induced hyper- and hypo-thyroidism.

2) In hyperthyroidism, besides tachycardias, marked decrease and finally complete disappearance of respiratory arrhythmias were noticed These can be looked upon as characteristics in hyperthyroidism.

3) In hypothyroidism, on the contrary, bradycardias were accompanied by exaggerated respiratory arrhythmias.

4) There was noticed a distinct correlation between the respiratory arrhythmias and pulse rates throughout the experimental conditions.

5) These changes in respiratory arrhythmia were found to be due mainly to changes in the expiratory pulse intervals, the inspiratory pulse intervals, on the other hand, being modified but little.

References.

1) Boas, and Weiss, Jour. Amer. Med. Ass., 1929, **92**, 2169.

2) Taguchi, Tohoku Igaku Zassi (The Journ. of Tohoku Medical Society), 1950, **39**, in press.

3) Matsuda, Tohoku Journ. Exper. Med., 1948, **49**, 246.

4) Palmer, Amer. Journ. Physiol., 1916–17, **42**, 572.

5) Anrep, Pascual and Rössler, Proc. Roy. Soc. B, 1936, **119**, 191 and 218.

6) Matsuda and Suzuki, Nihon Seiri-Gaku Zassi (Journ. of Japanese Physiol. Soc.), 1949, **11**, 19 (Society Proceeding).

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On the Patterns of Cardiac Rhythm in Healthy Men, with Especial Reference to the Slow Rhythm Simulating Traube-Hering Waves of Blood Pressure.

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Introduction.

While the heart rate of man measured by counting the pulse rate during a definite interval of time, say during one minute, is fairly constant under resting conditions, it is nothing more than an average of instantaneous cardiac rates which fluctuate from time to time over wider ranges than those of the average pulse rates. The most conspicuous of such fluctuations of the instantaneous cardiac rates are the respiratory arrhythmias which, having been long noticed, were studied by many researchers by means of the sphygmography or electrocardiography. Even by these methods of recording, however, it was not easy, or practically almost impossible to investigate accurately the changes of the instataneous cardiac rhythm continuously for longer intervals of time.

Recently Matsuda constructed a new type of instrument[1] which is especially suitable for such purpose. This is a modification of his *Cardiotachograph*[2] and depicts very clearly the time course of changes of the instantaneous cardiac rhythm.

During I was studying the human cardiac rhythm under resting conditions by means of this instrument, I noticed that the pattern of cardiac rhythm is, to some extent, characteristic to each individual. As some of the individuals have the features in common, it seems possible to classify the normal patterns of human cardiac rhythm into types. The present report aims to elucidate the components of the cardiac rhythm which characterize its patterns as the bases for the classification of its types.

Experimental.

Method.

Twenty healthy, adult individuals (15 men and 5 women, covering ages from 19 to 52 years) were selected for study. They were members of our laboratory or medical students and all were well familiarized to the examination by repeated experiences.

Recording of the cardiac rhythm by cardiotachograph was carried out on them under quiet, resting conditions between meals; usually in recumbent positions. At times observations in sitting or standing positions were performed, or during sleep if necessary.

Records from children or some patients (hyperthyroidism, cerebra tumors or epilepsy) were also taken for purposes of reference.

Results and Discussion.

In all cardiotachograms taken of normal persons I never failed to notice the respiratory arrhythmias, the degree of which, however, is variable from one individual to another. (Fig. 1). Concerning this type of arrhyth-

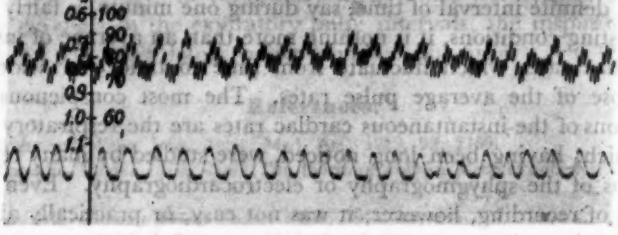

Fig. 1. Cardiotachogram (new type of recording) of man in quiet, recumbent posture. Upper: Time in two seconds, Middle: cardiotachogram. Heights of summits of wavelets show instantaneous pulse rate, numerical value of which can be read by scale on left. Numbers on the left side is pulse intervals in seconds. Those on the right side pulse rates in a minute. Note distinct respiratory arrhythmia. Lower: Pneumogram, inspiration upward (optical recording by Kato and Sato's method, Tohoku Journ. Exp. Med., 1950, **52**, 444).

mias it is not discussed here as detailed descriptions of them will be found elsewhere.[3-4]

Besides the respiratory arrhythmias there were usually found in the cardiotachograms various sorts of fluctuations of cardiac rates. Some of these fluctuations appeared fairly regularly in forms of slow waves recurring in succession and others were quite irregular. The more regular waves

could be identified by careful observation in the records of almost all individuals (Fig. 2), and may be regarded as one of the constant com-

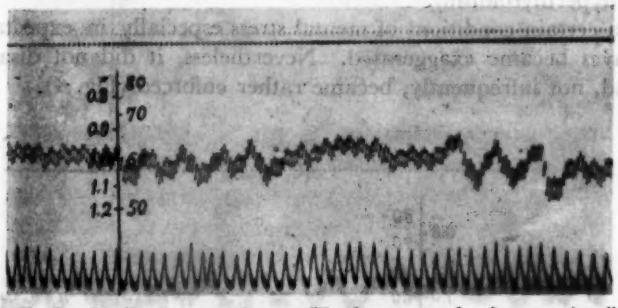

Fig. 2. Cardiotahogram of man. The slow waves take place occasionally in groups.

ponents in the human cardiac rhythm; I call it simply "*slow waves*". Such waves, indeed, have not escaped the observations of some investigators; especially Fleisch and Beckmann,[5] who studied of human cardiac rhythm by the use of Fleisch's *Pulszeitschreiber*, recognized and designated it as "*langsame Wellen*", that is slow waves.

The slow waves appeared in the records as oscillations waxing and waning slowly, being much longer than those associated with respiration. Periods of these waves were not necessarily constant among individuals ranging from 7 to 60 seconds. Even in the same subject the period of each wave was not definite. But the predominancy in period was usually between 10 to 15 seconds as represented in the frequency polygon in Fig. 3. These slow waves made their appearance ordinarily in groups, though they came and went spontaneously without any noticeable reason. The sizes of oscillations, that is the amplitudes of the slow waves were from 5 to 15 in heart rate per minute. The periods of the waves had no correlation to their amplitudes. It seemed that the development of the slow waves were not essentially influenced by the cardiac rate *per se*, though the waves became more apparent when there was a moderate tachycardia (e.g. in standing position), for the latter tends to

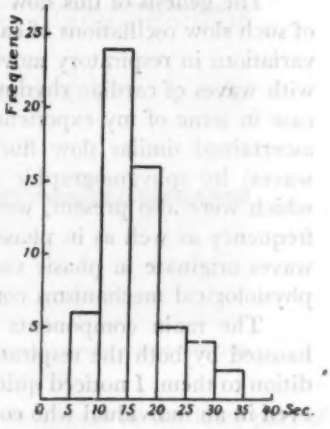

Fig. 3. Frequency of occurrence of the slow waves in respect to its period in an individual.

reduce the respiratory arrhythmia which often obscures the slow waves. In a marked tachycardia, however, the slow waves disappeared completely (e.g. in hyperthyroidism).

In a certain conditions of mental stress especially in expectation the slow waves became exaggerated. Nevertheless, it did not disappear in sleep and, not infrequently, became rather enforced (Fig. 4).

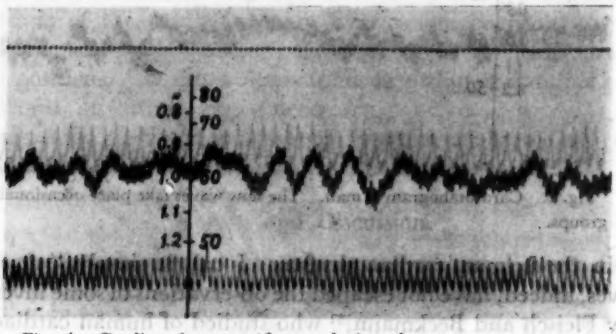

Fig. 4. Cardiotachogram of man during sleep.
The individual is the same as in Fig. 2. Note the marked similarity in pattern in both figures.

In patients of cerebral tumors or epilepsy there was nothing particular as to the slow waves.

The genesis of this slow wave is not yet clear. Cerletti, who noticed of such slow oscillations of cardiac rhythm in rabbits, ascribed it to phasic variations in respiratory movement that were seen to occur synchronously with waves of cardiac rhythm.[9] It is true also in man that such was the case in some of my experiences, but not always. In a few cases where I ascertained similar slow fluctuations of blood pressure (Traube-Hering waves) by sphygmographic recording, the slow waves of heart rhythm which were also present, were not related to the blood pressure waves in frequency as well as in phase. Though it is most probable that the slow waves originate in phasic variations in the tone of cardiac nerves, the physiological mechanisms concerned are to be determined.

The main components in the human cardiac rhythm are not exhausted by both the respiratory arrhythmia and the slow waves. In addition to them, I noticed quite irregular fluctuations, at times very marked, even in an individual who could be regarded to be in a completely relaxed condition physically as well as mentally (Fig. 5). In some individuals there were always observed such "*irregular waves*", which gave a characteristically irregular, zig-zag pattern to cardiotachogram. That these irregular waves were not necessarily due to mental restlessness as it might be

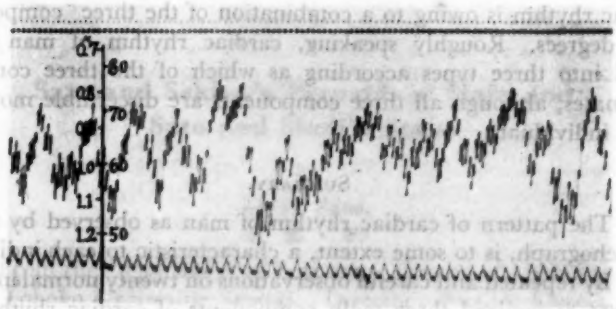

Fig. 5. Cardiotachogram of a young girl. Both the irregular waves and the slow waves are seen.

conjectured, could be recognized by a following fact: in one observation on a young girl, I ascertained that the irregular waves present did not disappear even when she was in sound sleep and persisted more or less throughout the sleeping hours. As a general rule, the irregular waves were seen more likely in younger than in older persons. In children the irregular waves were constant findings notwithstanding they were fully accustomed to the experiment. It is interesting, however, that the irregular waves and even the slow waves in children vanished from sight when they fell asleep (Fig. 6) leaving only very regular respiratory arrhythmias.

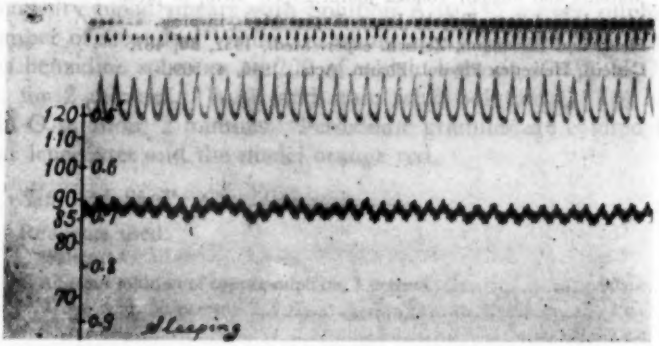

Fig. 6. Cardiotachogram of a child. Quite regular waves are respiratory arrhythmia. No other fluctuations are visible.

In the case of Fig. 6, the slow waves began to appear in the later part of sleep.

Taking the three components described above, viz., 1) the respiratory arrhythmias, 2) the slow waves and 3) the irregular waves into consideration, it became clear to me that the individual characteristic in the pattern

of cardiac rhythm is owing to a combination of the three components in various degrees. Roughly speaking, cardiac rhythm of man may be classified into three types according as which of the three components predominates, although all three components are discernible more or less in every individual.

SUMMARY.

1. The pattern of cardiac rhythm of man as observed by means of cardiotachograph, is to some extent, a characteristic to each individual.

2. By repeated and careful observations on twenty normal individuals there were recognized three main components of cardiac rhythm which characterized its pattern. That is 1) the respiratory arrhythmias, 2) the slow waves and 3) the irregular waves.

3. The features of these three components except the respiratory arrhythmias were described and discussed.

The expenses of this work were defrayed by a Grant from the Foundation for Promotion of Scientific Research of the Education Department of Japan.

K. Matsuda.

References.

1) Matsuda, Tohoku Journ. Exper. Med., 1950, **52**, 75.
2) Matsuda, Tohoku Journ. Exper. Med., 1948, **49**, 246.
3) Matsuda and Suzuki, Tohoku Journ. Exper. Med., in press.
4) Matsuda and Sato, Tohoku Journ. Exper. Med., in press.
5) Fleish und Beckmann, Zeitsch. exper. Med., 1932, **80**, 487.
6) Cerletti, Helvetica Physiol. Pharm. Acta., 1946, **4**, 589.

The Tohoku Journal of Experimental Medicine, Vol. 53, Nos. 3 & 4, 1951.

Sato and Sekiya's Peroxidase Stain and Sato and Shoji's Stain.

By

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(Received for publication, March 27, 1950)

In Blakiston's New Gould Dictionary (1949, 1st ed., p. 977) *Sato and Shoji's stain* appears, while there is no mention of *Sato and Sekiya's stain.* The latter reaction appears in a number of American and European books of hematology, also in Dorland's medical dictionary (in the latest editions).

A consise dictionary cannot do any more than to give just an idea of each entry; it is too much to ask of it a correct or precise description of each expression.

Here a correct description of both peroxidase reactions will be given.

1. *Sato and Sekiya's peroxidase stain:—*

Flood dry blood smears with Solution A (0.5% copper sulphate) for any number of seconds. Without washing apply Solution B (a saturated aqueous benzidine solution, to 200 of which 4 drops of 3% H_2O_2 were added), for 2 minutes. Wash and stain with Solution C (1% aqueous Safranin O solution) 2 minutes. Peroxidase granules are colored blue in granular leucocytes and the nuclei orange red.

2. *Sato and Shoji's peroxidase stain.*

A. Reagents used.

1. Solution A.

Aqueous solution of copper sulphate, 1 percent	90 c.c.
Acetic acid, 30 percent	1 c.c.
Glycerin	2 c.c.
Distilled water up to	300 c.c.

Filter.

2. Solution B. and Solution C.
Quite the same as these of Sato and Sekiya's method.

B. Method used.

Draw blood into the pipette up to the 0.3 mark and after rapidly wiping the end clean, immerse it at once into Solution A plus safranine (add two drops of Solution C to 2 c.c. of Solution A). Draw up this solution until the mixture fills the lower third

or fourth of the bulb of the pipette. Hold a finger over the end and rotate the pipette on its long axis. This causes mixing and avoids the formation of air bubbles. Wait for 2 minutes and then fill to the 11.0 mark with Solution B. Insure thorough mixing by rotation. The fluid is then introduced into the counting chamber.

Result: (Sato and Shoji's stain)

Myeloid leucocytes are stained greenish blue. Monocytes appear faintly red with distinct blue granules. Lymphocytes are stained distinctly red without any shade of blue or green.

The use of this method for the white cell count in daily routine work may lead to an unexpected discovery of a case of agranulocytosis or one with the striatal blood symptom.

Sato and Shoji's stain is thus a counting chamber method for Sato and Sekiya's stain.

In short, Sato and Sekiya's stain is a *dry* smear method, while Sato and Shoji's stain is a *humid* method.

References.

1) Sato, A. and Shoji, K., J. Laborat. and Clinic. Med., 1928, **13**, 1058; Tohoku Igaku Zassi, 1929, **9**, 262.

2) Tokue, K. and Yasumoto, M., Am. J. Dis. Childr., 1929, **38**, 1037.

3) Sato, A. and Yoshimatsu, Sh., Am. J. Dis. Childr., 1925, **259**,301; Sato, A. and Kuribayashi, S., Tohoku J. Exp. Med., 1939, **35**, 522.

The Tohoku Journal of Experimental Medicine, Vol. 53, Nos. 3 & 4, 1951.

ABO-Blood-Group Lipoids in Human Liver.

First Report.*

By

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(Received for publication, March 27, 1950)

From human livers dehydrated with cold alcohol, lipids extractible with boiling alcohol and ether were separated and therefrom by the aid of organic solvents a group-active fraction was obtained. Though devoid of an affirmative evidence, I assumed the products pretty pure and examined their properties and composition. The present account deals with the preparation procedure and analytical results. Shortage of material did not allow sugar determination in the Group B lipoid but it appeared that A, B and O group lipoids from human liver contain generally galactose, hexosamine, P and N in approximate equivalent proportions of 1:1:10:10 and possess minimum molecular weight of about 6500 (computed from the galactose content). N.E.=600~700. Nitrogen proved to be partially present in the form of primary amine.

The group lipoids here showed similar solubility and qualitative reactions as those from a liver metastasis of gastric cancer (Aikawa[1]) and erythrocytes (Hamasato[2]) despite of a different composition.[3]

Experimental.

Preparation of the Group Lipoids.

The group lipoids were separated, taking the group activity as an indication, by a procedure resembling those of the predecessors in our Institute. A typical example will be given below.

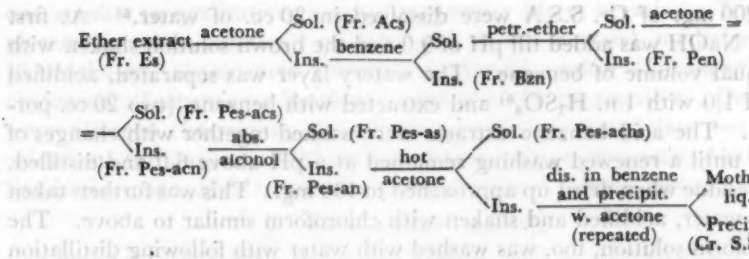
Livers from two persons[4] belonging to Group A, weighing 2000 g. in total, were cut into small pieces, washed with physiological saline to remove blood and bile and kept in 5 volumes of alcohol (95%) for about a month. The tissue was then squeezed and ground in a masticator. The obtained pulp was boiled with 3 volumes of alcohol (95%) for 4 hours under reflux.

* 4th report of Masamune and co-workers' "Chemistry and Biology of Lipids."

While hot the extract was separated by decantation and by means of a press. The residue was subjected again to extraction with 2 volumes of alcohol (95%) in a like manner and then, after drying up in a Faust apparatus, pulverized in a mortar and treated with ether (200 cc.) in a Soxhlet apparatus exhaustively. The alcoholic extracts were filtered from yellowish flocculent precipitates, that had come out on standing, and distilled in vacuo as far as possible. The syrupy residue changed mucilaginous after long time (about 2 weeks) in a vacuum desiccator (H_2SO_4), whereupon it was also treated with ether. The ether extracts were combined and distilled in vacuo and dried as above. The remaining dark brown gelatinous substance amounted to 28 g.—Fr. Es.

Fr. Es was washed with six 40 cc. portions of cold acetone. And the part undissolved and that dissolved and distilled were both placed in a desiccator. The latter—Fr. Acs—remained as a viscous brown fluid. The former—Fr. Acn—was hygroscopic and brown, gelatine-like and weighed 22 g. This was extracted with benzene (Five 20 cc. portions of benzene employed). The insoluble substance here—Fr. Bzn—was colored grayish brown and not more than 0.1 g. (96 mg.). The benzene solutions were evaporated together in vacuo with following drying (20 g. of a brown solid were left) and removed from a petroleum-ether insoluble substance (a trace—Fr. Pen). Glistening dark brown gelatine-like substance obtained was washed with acetone thoroughly and dried—Fr. Pes-acn. The yellow colored washings left a residue on distillation—Fr. Pes-acs. Fr. Pes-acn and Fr. Pes-acs were dark brown and yellowish respectively. Fr. Pes-acn (15 g.) was further subdivided into the absolute alcohol-soluble and -insoluble portions. The solvent was changed many times until no coloring matter came out. The insoluble (6 g. when dry)—Fr. Pes-an—was yellowish non-sticky powder. The soluble (Fr. Pes-as) weighed 8 g. after expelling off the solvent.

In the next Fr. Pes-an was kneaded with 30 cc. of acetone. Since, here, acetone did not get colored, the mixture was boiled under reflux for 2 hours and centrifuged while hot. The yellowish supernatant liquid gave 150 mg. of brown resinous substance on vacuum distillation and drying. The centrifugate was dried and after taking up in 30 cc. benzene (The whole substance went into solution), 5 volumes of acetone added. Yellowish brown precipitates came out. Centrifuged. The residual after distillation of the supernatant fluid (yellowish) was again dissolved in 10 cc. of benzene and precipitated with acetone (50 cc.). Centrifugation, distillation of the supernatant liquid, dissolution of the remainder and precipitation was again repeated. This time precipitates were few (The mother liquid was almost non-colored). The three successive lumps of precipitates were united and dried (Cr. S.S.A, 2.3 g.).



Final purification was carried out by transfer of the substance from water to organic solvents:—

Table I.

Occurrence of Agglutination of Erythrocytes by Normal Antisera Treated with Fractions of a Lipid-mixture (obtained with boiling alcohol and ether) from Livers of Group A Individuals.

(– no agglutionatin, ± faint aggl., + slight aggl., ++ marked aggl., +++ strong aggl.)

Fraction	Group of serum	Dilution of fractions							
		1 % Original	×2	×5	×10	×1.6 ×10	×3.2 ×10	×6.4 ×10	×1.28 ×10²
Es	A	++	++	++	++	+++	+++		
	B	–	–	+	++	+++	+++		
Acs	A	+++	+++	+++	+++				
	B	+++	+++	+++	+++				
Bzn	A	+++	+++	+++					
	B	+++	+++	+++					
Pen	A	+++	+++	+++					
	B	+++	+++	+++					
Pes-acs	A	+++	+++	+++					
	B	+++	+++	+++					
Pes-as	A	+++	+++	+++	+++				
	B	±	++	+++	+++				
Pes-an	A	+++	+++	+++	+++	+++			
	B	–	–	–	+	++			
Pes-achs	A	+++	+++	+++					
	B	+++	+++	+++					
Cr. S.S.A	A	+++	+++	+++	+++	+++	+++	+++	
	B	–	–	–	±	++	+++	+++	
S.S.A	A	+++	+++	+++	+++	+++	+++	+++	+++
	B	–	–	–	–	–	–	±	+++
(Pes-as)′	A	++	+++	+++	+++				
	B	±	++	+++	+++				

200 mg. of Cr. S.S.A were dissolved in 20 cc. of water.[5] At first 1.0 n. NaOH was added till pH of 9.0 and the brown solution shaken with an equal volume of benzene. The watery layer was separated, acidified to pH 1.0 with 1 n. H_2SO_4[6] and extracted with benzene (two 20 cc. portions). The acid benzene extracts were washed together with changes of water until a renewed washing remained at a pH above 6.0 and distilled. The residue when dried up approached to 130 mg. This was further taken up in water, acidified and shaken with chloroform similar to above. The chloroform solution, too, was washed with water with following distillation to dryness. 82 mg. of substance were yielded—S.S.A. The product inhibited isoagglutination of A erythrocytes at a dilution of 1:3200 (s. below).

Fr. Pes-as, whose 1% solution was faintly group-active, was also treated with boiling acetone, and from the undissolved fraction the part soluble in benzene and precipitable with acetone was separated—Fr. (Pes-as)′ (yellowish non-sticky powder). The attempt, however, failed to raise the potency.

Anti-isohemoagglutinative Potency of the Lipid Fractions.

Table I shows the serological potencies of the lipid fractions in the run taken as an example of preparation. The assay was effected as by Hamasato[2], but Cr. S.S. and S.S. were dissolved in physiological saline without the use of alkali. Regarding the corresponding fractions from the liver of a Group B person, of which the active ones were B specific, the results were resembling, too. The dilution of A- and B-S.S.'s, whereat the inhibition of the isoagglutination manifested itself, varied from 1:1600 to 1:3200. The lipid mixture from the liver of an O person gave no fraction reactive to A or B serum.

Physical and Chemical Properties of S.S.'s.

1) Solubility.

S.S.'s had similar solubility irrespective of their blood group. (Table II)

TABLE II.

Solubility of Group Lipoids (S.S.'s) from Human Liver.

(− insoluble, + partly sol., ++ mostly sol., +++ completely sol. "hot" signifies "heated just to boiling," except when glacial acetic acid was employed.)

Temp.	Solvent									
	Water	Methanol	Ethanol	Ether	Petr.-ether	Acetone	Chloroform	Benzene	Tetralin	Glacial acetic acid
Cold	+++	−~+	−	−	−	−	+++	+++	+++	+++
Hot	+++	+~++	+	−	−	−	+++	+++	+++	+++ (100°C)

2) Qualitative tests.

All the S.S. preparations belonging to different blood groups resembled

one another in qualitative chemical property. Biuret negative. Tests for glycerol (acrolein reaction), pentose (with aniline acetate), methylpentose (Rosenthaler), uronic acid (Neuberg-Saneyoshi), sulfur (Lassaigne) and cholesterol (Liebermann-Burchard) also resulted in negative. Detected were nitrogen (Lassaigne), phosphorus (ammonium molybdate test with oxidation), ordinary sugar (Molisch) and amino sugar (Hamasato-Akakura). Saccharose-conc. H_2SO_4 test (Pettenkofer) gave a purple colour.

3) Quantitative analysis.

In addition to analytical figures of nitrogen (micro Kjeldahl), phosphorus (Haneda, Sinokawa and Yasuoka's modification of Plimmer method) and ash (Pregl), are given in Table III the colorimetric results, expressed as galactose, of immediate reactions of the substances with indole (Dische), orcinol (Ozaki's modification of Tillmans and Philippi method) and α-naphthol (Reaction I of Dische). Galactose can be accurately determined by the aid of these reagents only after acid hydrolysis of the substances with following removal of water-insoluble components and phosphotungstic acid-precipitable ones. Thus the values in the table are remote from the actual, being much larger, but they will serve the purpose of comparison with the corresponding values in those investigations[1)2)] when we were

TABLE III.

Composition of Group Lipoids (S.S.'s) from Human Liver.

(Figures express percentages.)

Preparation	Analysis					
	Direct colour development as galactose			Phosphorus	Nitrogen	Ash
	Indole r.	Orcinol r.	α-Naphthol r.			
S.S.A Prep. I	9.4	7.3	8.0	4.4	1.86	5.9
S.S.A Prep. II	9.2	6.8		4.1	1.84	6.5
S.S.A Prep. III	9.0	6.1		4.0	1.88	6.0
S.S.B Prep. I	9.4	6.9		5.4	2.24	5.2
S.S.O Prep. I	8.3	5.0		5.2	2.10	4.8
Average	9.1	6.4		4.6	1.98	5.7

unaware of the mentioned fact. The table indicates that, at least so far N, P and the components reacting to indole and orcinol concern, the S.S. preparations all coincided mutually and the equivalent ratio of the average values of N and P was 1:1.

As will be seen in a forthcoming report,[7)] I have isolated galactose and glucosamine from the present A group lipoid after hydrolysis. Thereby 2.7% galactose and 2.0% glucosamine were obtained. On the other

hand, Masamune-Tanabe procedure[8] for fractionated analysis of so-called glycoproteins was adapted[9] to a preparation of the Group O lipoid (S.S.O Prep. I) except which no enough material was available, and the hydrolysate-fractions exhibited reduction and coloration corresponding to 2.5% galactose and 2.0% hexosamine. Not only from these data but also from previous findings in group carbohydrates it is regarded as justified to conclude that A, B and O group lipoid from the same organ or tissue are not divergent in galactose and hexosamine content. Furthermore, the analysis and isolation of hexosamine above are rather accepted to have given lower values than the virtual and it is assumed that in fact the materials involved an amount of hexosamine equimolecular to galactose, because acid hydrolysis gave in both cases an voluminous insoluble mass involving palmitic acid and phosphate rest to which adsorption of a part of the hexosamine probably occurred.

In short, in group lipoids of human liver hexosamine, galactose, P and N are considered to be present in approximate equivalent ratios of 1:1:10:10 (basis of calculation: 2.6% galactose (average value), 2.6% hexosamine, 4.6% P, 1.98% N, 5.7% ash (s. the table)). Calculated from the galactose content the minimum molecular weight approximates to 6500 $[(100/2.6\times\frac{100}{94.3})\times 180=6528]$.

Amino nitrogen (micro Van Slyke) amounted to 1.26% in S.S.A Prep. I and 0.79% in S.S.B Prep. I.

S.S.A, Prep. I.
100 mg. substance, 0.21 cc. N_2 (11°C, 770 mm. Hg). Found, 1.26%.
S.S.B, Prep. I.
11.32 mg. substance, 0.15 cc. N_2 (11°C, 770 mm. Hg). Found, 0.79%.

Whether free amino-nitrogen varies in amount according to the blood group or the discrepancy is due to contaminants will be settled by a future study.

Neutral equivalent. It was measured by formol titration. 3 cc. of a solution were titrated with 0.01 n. NaOH, and the total alkali used before and after addition of dilute neutral formalin (three times diluted formalin was neutralized to phenolphthalein and 3 cc. of it were added) was taken for the calculation. The blank experiment was made in parallel.

S.S.A, Prep. I.
$$\text{N.E.}=\frac{0.0207\times 1000}{3.13\times 0.01}=661 \text{ (703 on ash-free basis).}$$
S.S.B, Prep. I.
$$\text{N.E.}=\frac{0.0236\times 1000}{3.75\times 0.01}=629 \text{ (664 on ash-free basis).}$$

SUMMARY.

1. From human liver group active lipoids were separated. Of them A and B group substance inhibited isoagglutination of erythrocytes at a dilution from 1:1600~3200 specifically.

2. These group lipoids dissolved in water, benzene, chloroform, tetralin and glacial acetic acid, but not in ethanol, ether, petroleum ether and acetone. They dissolved in methanol scarcely in the cold but to a certain extent when boiled.

3. The group lipoids contained galactose, hexosamine, P and N in equivalent proportions of roughly 1:1(most probably):10:10. Their minimum molecular weight (The substances were presumed as nearly pure) of 6500 was computed out from galactose content. N.E., 600~700.

The costs of this work were defrayed by a grant from the Education Department through the Grant Committee of National Research Council, which is gratefully acknowledged.

H. Masamune.

References and Notes.

1) Aikawa, Tohoku J. Exp. Med., 1949, **51**, 367.

2) Hamasato, ibid., 1950, **52**, 17.

3) Aikawa and Hamasato estimated galactose in group lipoids by direct application of colour reactions to the substances. But group lipoids involve components which influence the results enormously, so that the galactose values of these authors are not reliable. The equivalent ratio of P to N was 1:3 in the group lipoid from the liver metastasis of gastric cancer and 2:3 in the ones from erythrocytes. Regarding the content of the components see the original papers.

4) Livers were afforded from the Institute of Forensic Medicine (Director: Prof. T. Murakami) where the autopsy had been made.

5) A perfect solution was given.

6) The solution was completely precipitated at pH 1.0.

7) Matuda, Tohoku J. Exp. Med., in press.

8) Masamune and Tanabe, J. Bioch., 1938, **28**, 19.

9) 130 mg. of the substance were hydrolyzed with 10 cc. of 1 n. H_2SO_4 and a 1 and a 5 cc. portion of the hydrolysate were used respectively for the total-sugar (galactose and glucosamine) and galactose determination.

Summary.

From human liver group active lipoids were separated. Of them A and B group substance inhibited hemagglutination of erythrocytes in dilutions from 1:1600–3200 specifically.

These group lipoids dissolved in water, benzene, chloroform, tetralin and glacial acetic acid, but not in ethanol, ether, petroleum ether and acetone. They dissolved in methanol scarcely in the cold but to a certain extent when boiled.

The group lipoids contained galactose, hexosamine, P and N in equivalent proportions of roughly 1 (more probably 1:0.9:1). Their minimum molecular weight (the substances were presumed as nearly pure) of 6300 was computed on basis galactose content.

The roots of this work were delivered by a grant from the Rockefeller Foundation through the Swiss Committee of National Research Council, which is gratefully acknowledged.

References and Notes.

1) Adlers, Tiselius and Thunberg, Biochem. Z. 1937, 291, 31.
2) Thunberg, ibid., 1936, 285, 17.
3) Adlers and Thunberg extracted substance in group lipoids by Avoid emulsions of other fractions in the substance. The group lipoids insoluble in ether, petroleum ether, chloroform, benzene, tetralin, glacial acetic acid, but soluble in ethanol, methanol, water and pyridine. The group lipoids contained galactose, hexosamine, P and N in equivalent proportions of roughly 1 (more probably 1:0.9:1). Their minimum molecular weight (the substances were presumed as nearly pure) of 6300 was computed on basis galactose content.
4) Adlers, Tiselius and Thunberg, Biochem. Z. 1937, 291, 31.
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Color Processes and Physiological Induction in Frog's Retina.

By

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Motokawa[1)-6)] studied various phenomena of vision by means of his method which is based on the fact that the electrical excitability of the retina changes after cessation of illumination with a time course characteristic of the wave-length of the light used for illumination. In his experiments on human eyes the index of excitation for determination of electrical excitability was a just perceptible electrical phosphene. In the present investigation similar experiments were performed on excised frog eyes with action potentials of optic nerves as the index in order to confirm the objectivity of the phenomena observed on human eyes.

Experimental.

Method and Procedure.

As experimental material, eyes of Japanese water frogs (Rana nigromaculata) with their optic nerves intact were used. The experimental arrangements used in this experiment are shown schematically in Fig. 1. As can be seen in the figure, non-polarizable electrodes of Zn-$ZnSO_4$-gelatine type were placed on the cornea and on the posterior pole of the eye-ball in such a manner that the stimulating current might flow through the eye-ball from the retina to the cornea. The stimulating current consisted of a single constant current pulse of 1 sec. in duration. The action potentials of the optic nerve were led off by means of a pair of non-polarizable electrodes of Ag-AgCl type to a four stage R-C-coupled amplifier, whose time constant was 0.25 seconds.

Since a good contact between the nerve and the electrodes was a necessary condition for stable functioning of the amplifier, the contact was secured by interposing a pad of Ringer-soaked cotten between the nerve and the electrode. The action potentials were observed on a screen of a cathode ray oscillograph. Shock artefacts could not completely be avoided, but determination of thresholds was hardly disturbed by them, for it was

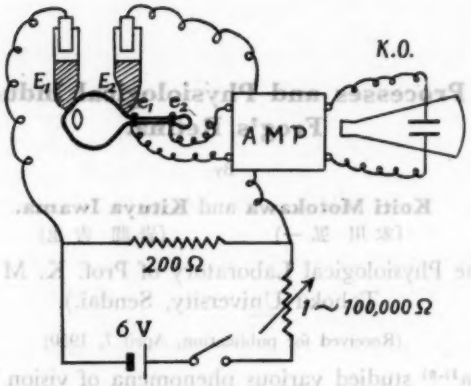

Fig. 1. Arrangements. AMP: amplifier. E_1 and E_2: stimulating electrodes of Zn-$ZnSO_4$-gelatine type. e_1 and e_2: leading-off electrodes of AgCl type. K.O.: cathode ray oscillograph.

easy to distinguish action potentials from shock artefacts.

After a preliminary dark adaptation of about 15 minutes, the resting level of the electrical threshold at make was determined. Threshold voltages were usually of the order 0.1 volt. Then, the eye was exposed to light for 4 seconds, and after cessation of illumination electric stimuli of varying strengths were delivered every 30 seconds, till about 10 minutes after removal of the light-stimulus. We endeavored in this series of electrical stimulation to adjust the intensity of each stimulus so that the stimulus might cause just perceptible action potentials. If any test-stimulus evoked no response or too strong action potentials it was tried in the second series with an increased or decreased stimulating voltage. When the same procedure of experimentation—pre-illumination and then electrical stimulation at intervals of 30 seconds—is repeated a few times, successful trials become numerous enough to construct an excitability-time curve with the data so obtained.

In order to express data quantitatively, a quantity ζ was introduced, which represents a percentage increase of electrical excitability (reciprocal of threshold) above the resting level.

Results.

1. *Three Kinds of Color Processes.*

When white light is used for pre-illumination a ζ-t curve or an excitability curve is obtained, which shows usually three humps at about 1.5, 3 and 5 minutes after the end of the illumination. These elevations may

be denoted by R, G and B (see Fig. 4). In some cases, however, only two distinct elevations are seen in the curve, resulting presumably from fusion of G and R elevations (see Fig. 3). The time from the end of pre-illumination to the maximum of each elevation was found to depend upon temperature; when room temperature lies between about 15 and 20°C we obtain crest times of the order mentioned above. In winter, however, the time course of each elevation was so much prolonged that the crest times were found longer than twice as long as those given above.

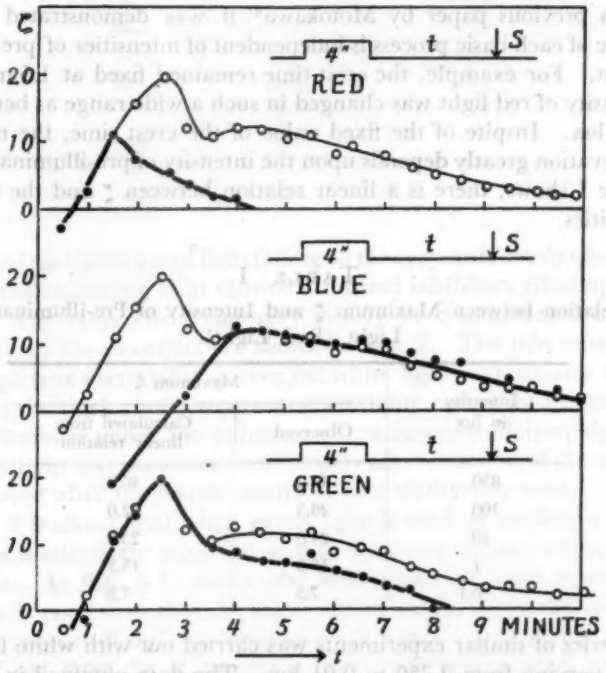

Fig. 2. Excitability curves for red, green and blue lights (thick curves). Thin curves connecting empty circles are excitability curves for white light. Time sequence of stimulation is given in insets. S: electric stimulus.

In Fig. 2 excitability curves obtained by pre-illumination with red, green and blue lights are illustrated in comparison with curves for white light. The colored lights were prepared by filters whose range of transmission was 620–740 mμ for red light, 470–550 mμ for green light and 410–490 mμ for blue light. As can be seen in the figure, the crest time of the curve for red light is about 1.5 minutes. The curve for blue light has a crest time of about 4.5 min. and coincides with the B elevation of the

curve for white light. The curve for green light differs from that for white light only in the part of the B elevation. All these findings indicate that there take place three color processes in the illuminated retina, and that their relative magnitudes are different according to colors. The R process is excited by red light, and the B process by blue light. Green light excites most strongly the G process, but can, to some extent, act upon the other processes.

2. *Relation between ζ and the Intensity of Light.*

In a previous paper by Motokawa[6] it was demonstrated that the crest time of each basic process is independent of intensities of pre-illuminating light. For example, the crest time remained fixed at 1.5 min. when the intensity of red light was changed in such a wide range as between 850 and 0.1 lux. Inspite of the fixed value of the crest time, the magnitude of the elevation greatly depends upon the intensity of pre-illuminating light. As Table I shows, there is a linear relation between ζ and the logarithm of intensities.

TABLE I.

Relation between Maximum ζ and Intensity of Pre-illuminating Light (Red Light).

Intensity in lux	Maximum ζ	
	Observed	Calculated from linear relation
850	41.0	40.0
100	28.5	32.0
10	24.0	23.5
1	15.5	15.5
0.1	7.5	7.5

A series of similar experiments was carried out with white light of intensities varying from 2,750 to 0.01 lux. The data obtained in this series of experiments are shown in Table II. As can be seen in this table, the linear relation holds in the intensity range from 2,750 to 10 lux. Below 10 lux, however, the observed values are generally greater than the calculated values. A similar deviation from the linear relation was observed also in the human experiments reported previously,[7] and we ascribed the two parts linear and non-linear of the curve to different mechanisms, cones and rods.

3. *Experiments of Retinal Inhibition.*

As has been reported by Motokawa,[1] it was found in a human

TABLE II.

Relation between Maximum ζ and Intensity of Pre-illuminating Light (White Light).

Intensity in lux	Maximum ζ	
	Observed	Calculated from linear relation
2750	42.5	43.0
750	37.0	36.8
100	26.0	26.5
30	20.0	20.5
10	14.5	14.5
1	8.5	3.0
0.3	8.5	—
0.1	8.5	—
0.01	6.5	—

experiment that weak green light falling on the eye continuously after removal of the pre-illuminating light showed a distinct inhibitory effect upon the G process. The same effect can very clearly be demonstrated on excised frog eyes. A few examples are shown in Fig. 3. The thin curves in this figure represent excitability curves for white light without any inhibitory light, and the thick curves represent excitability curves for the same white light determined under the influence of continuous inhibitory light. The inhibitory light was presented immediately after cessation of the white light and removed after the test of electrical excitability was over.

Fig. 3 B shows that when green light is used as inhibitory light the G process is selectively inhibited so that the curve consists of the R and B elevations. As Fig. 3 C shows, the blue inhibitory light selectively inhibits the B process so that the curve shows only a single elevation which evidently consists of the two components R and G. Under the inhibitory action of red light the R process is most severely inhibited, and the G process is inhibited only moderately, while the B process remains uninfluenced. These selective inhibitory actions may be accounted for in the following manner: In general, excitability curves represent an electrical supernormal phase following a refractory phase which is caused by an illumination of the retina. When the retina is continuously exposed to inhibitory colored light some retinal elements cannot recover from their refractoriness. Consequently no supernormal phase appears at least for the element involved, with the result that they drop out of our excitability curve.

From this consideration it follows that any colored light inhibits the

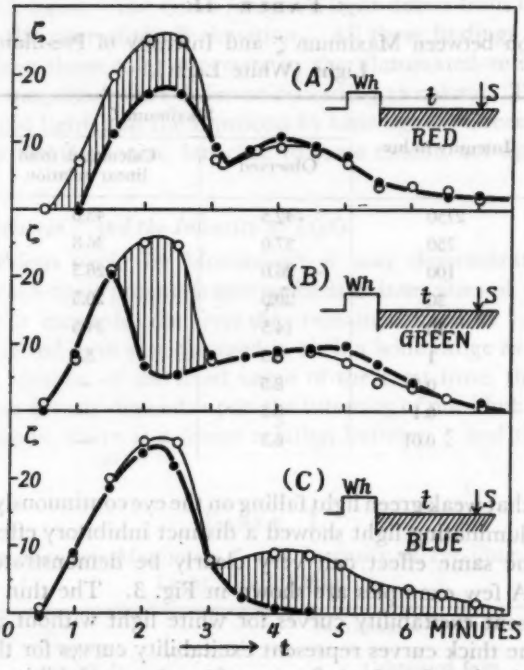

Fig. 3. Retinal inhibition. Thick curves represent inhibited curves for white light, and thin curves control without inhibition. In insets Wh represents white light, shaded parts indicate continuous presentation of inhibitory light, and S electric stimulus.

very element which it can excite. This principle is evidently exemplified in our experiments; for example, blue light evokes the B process when it is used as pre-illuminating light, and inhibits the same process when it is used as inhibitory light.

4. *Experiments of Retinal Induction.*

If pre-illuminating white light is preceded by any colored light the excitability curue for white light always suffers a remarkable change, and this change cannot be explained by summation of the effects of the two light-stimuli, colored and white. The curve so obtained is in general complementary in chracter to the curve which would be obtained by pre-illumination with the colored light alone. A few examples are shown in Fig. 4. In this figure, the thin curves connecting empty circles represent the excitability curves for white light alone, and the thick curves connecting solid circles indicate the excitability curves for the successive

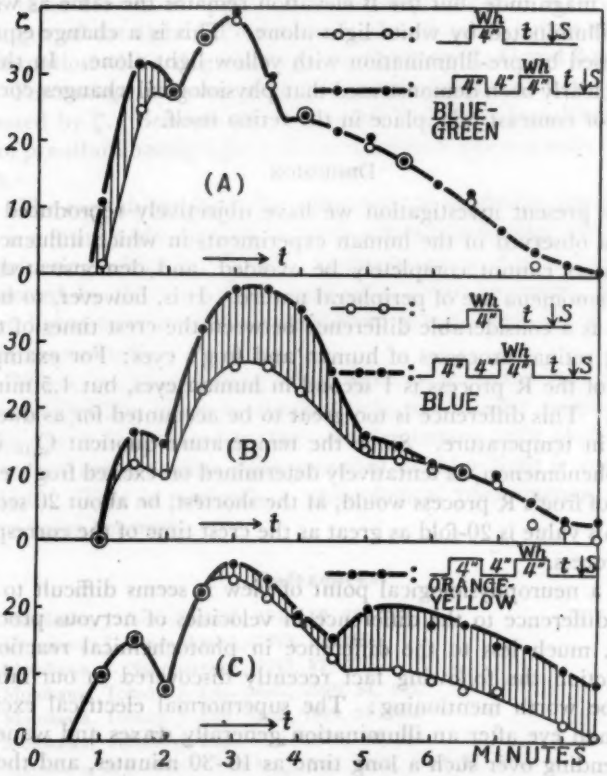

Fig. 4. Retinal induction. Thin curves represent excitability curves for white light alone, and thick ones the effects of successive stimuli, colored and white. Time sequence of light- and electirc stimuli is shown in insets.

light-stimuli, colored and white.

In the case (A) the preceding bluish-green light caused a remarkable increase in the magnitude of the R elevation, while no perceptible change whatsoever took place in the other elevations. This finding may be so interpreted that the successive stimuli acted as a whole like unsaturated red light, because this light gives rise to an excitability curve with a predominant R elevation, when it is used alone for pre-illumination. We know that when we look at a white or grey surface after having gazed at a bluish-green object, the surface looks tinged with red. It is now obvious that the phenomenon mentioned above represents a physiological counterpart of the psychological phenomenon called successive color contrast. When blue light is used as preceding colored light, the R and G elevations

increase in magnitude, but the B elevation remains the same as when the eye is pre-illuminated by white light alone. This is a change equivalent to that caused by pre-illumination with yellow light alone. In this manner, it has clearly been demonstrated that physiological changes corresponding to color contrast take place in the retina itself.

Discussion.

In the present investigation we have objectively reproduced all the phenomena observed in the human experiments in which influences from higher centers cannot completely be avoided, and demonstrated clearly that the phenomena are of peripheral nature. It is, however, to be noted that, there is a considerable difference between the crest times of the corresponding retinal processes of human and frog's eyes: For example, the crest time of the R process is 1 second in human eyes, but 1.5 minutes in frog's eyes. This difference is too great to be accounted for as due to any difference in temperature. Since the temperature quotient Q_{10} is about 2 for this phenomenon, as tentatively determined on excised frog's eyes, the crest time of frog's R process would, at the shortest, be about 20 seconds at 37°C. This value is 20-fold as great as the crest time of the corresponding human process.

From a neurophysiological point of view it seems difficult to ascribe this great difference to the difference in velocities of nervous processes in the retina, much less to the difference in photochemical reactions. In this connection the following fact recently discovered in our laboratory seems to be worth mentioning: The supernormal electrical excitability of the human eye after an illumination generally waxes and wanes a few times, extending over such a long time as 10–30 minutes, and the period of such waxing and waning is more prolonged with the time elapsed after withdrawal of the pre-illuminating light. The human excitability curve with which we have been dealing, is therefore nothing but the initial part of such a long lasting excitability change. If we take the second rise of electrical excitability into account we would obtain crest times of the same order as those of frog's excitability curves.

Summary.

The change in electrical excitability of excised frog eyes was investigated with action potentials of optic nerves as the index of excitation.

1. The excitability-time curve or excitability curve after an illumination by white light shows three humps at about 1.5, 3 and 5 minutes after the end of the illumination at room temperature of 15–20°C. These humps are called R, G and B elevations.

2. When colored light is used instead of white light for pre-illumina-

tion the three elevations appear in different proportions according to the wave-length of the light. Predominant elevations of curves obtained by pre-illumination with red, green and blue lights are R, G and B respectively.

3. Percentage increases of electrical excitability above its resting level are denoted by ζ. Between the maximum ζ and the logarithm of the intensity of pre-illuminating light a linear relation holds in a wide range of intensities.

4. The excitability curve for white light is markedly deformed when the eye is continuously exposed to weak colored light after removal of the pre-illuminating white light. This phenomenon is due to an inhibitory effect exerted by the continuous light upon color processes. Red, green and blue lights have a selective inhibitory action upon the R, G and B processes respectively.

5. When pre-illuminating white light is preceded by any colored light, an excitability curve is obtained which is similar to that obtained when the eye is illuminated by the complementary color alone. For example, in this respect white light preceded by blue light is equivalent to yellow light. This is evidently a physiological phenomenon underlying successive color contrast.

References.

1) Motokawa, J. Neurophysiol., 1949, **12**, 291.
2) Motokawa, J. Neurophysiol., 1949, **12**, 465.
3) Motokawa, J. Neurophysiol., 1949, **12**, 475.
4) Motokawa, Tohoku J. Exp. Med., 1949, **51**, 145.
5) Motokawa, Tohoku J. Exp. Med., 1949, **51**, 165.
6) Motokawa, Tohoku J. Exp. Med., 1949, **50**, 258.
7) Motokawa, and Iwama, Tohoku J. Exp. Med., 1949, **51**, 155.

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On the Neurinoma Fibril.

(A Contribution to the Study of Argyrophil Fibers)

By

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On classification of a tumor, detection of the argyrophil fiber (Gitterfasern") lying in the interstice, is a powerful criterion of differential diagnosis at present.

In spite of vigorous discussion on the argyrophil fiber in recent papers, it has been rather neglected, that the silver reaction itself depends upon chemical conditions of the tissue element. On the viewpoint of silver impregnation, a ready paraffin section would not always be equivalent to a frozen section evidently.

Bleaching plan by means of potassium permanganate and oxalic acid would also influence the argyrophil manner of tissue elements profoundly. The purpose of this paper consists in the recognition of the argyrophil character of the neurinoma fibril under various conditions. Comparing with a nasopharyngeal fibroma, writers referred to morphologic features of the neurinoma fibril as a tonofibril of ectodermal origin and to a fibroblastic metaplasia of the Schwannian cell.

Experimental.

Material and Methods.

(1) An walnut sized, acoustic neurinoma, found in the right cerebellopontile angle of a man, aged 58, who died of cerebral softening (Autopsy No. 31, 1941). The material has been preserved in Kaiserling's solution. Histologically, the tumor revealed a fasciculated appearance with the characteristic regimentation of the nuclei and a reticular zone partially.

(2) A naso-pharyngeal fibroma as a contrast, of the size of a dove egg, fixed in 10% formalin, was obtained surgically from a man, 18 years of age, in the oto-rhino-laryngologic clinic (Director: Prof. Tsuiki) of our University Hospital.

Both specimen, cut in a paraffin and a frozen section, was impregnated

by Bielschowsky, Perdrau, Foot, and Gross-Goldin's modification.

Results.

1) *Impregnation of a frozen section after Bielschowsky-Maresch* (1905) (*no use of potassium permanganate and oxalic acid*).

In the fasciculated area of neurinoma, there was rich production of a hair-like silver fibril running parallel with the nuclei of the tumor cells; no reticular formation of the fibril. The lanuginous feature of the fibril was prominent at the non-nucleated zone of the palisaded portion (Fig. 1). Cross section of the fiber bundle did not reveal any circular contour of the Schwannian cytoplasm, presenting an aggregation of dusty dotts or granules solely.

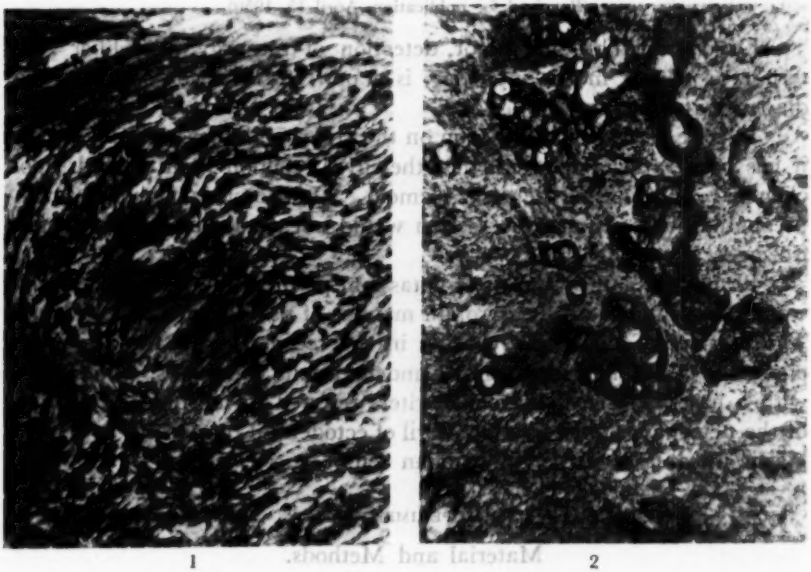

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Fig. 1. Palisaded area of a neurinoma (Bielschowsky-Maresch technique, frozen section).

Fig. 2. Angiomatous proliferation in a neurinoma (Perdrau technique, frozen section).

There was no positive proof of an intracellular silver fibril. In fibroma, on the other hand, rich collagenous fibers interlacing with a broad ribbon-like impression (Nageotte) presented a purple tone. A small nerve bundle enveloped with the perineurium was seen occasionally.

2) *Impregnation of a frozen section after Perdrau's method* (*passing through potassium permanganate and oxalic acid prior to the silver bathe*).

As the result of suppression of nuclear impregnation, the fasciculated area of neurinoma was seen to be composed of a long slender fibril solely; there was no characteristic regimentation of the nuclei.

Argyrophilic capillary tube ("Gitterrohr") was demonstrated very sharply (Fig. 3), although there was no direct communication of the two fiber systems. In fibroma, no marked difference was seen, compared with the previous method.

3) *Impregnation of a paraffin section in the same manner (Bielschowsky-Foot).*

The neurinoma fibril seemed to be thin and densly, like curly hair. Owing to expansion and contraction, the specimen tends to detach from the slide frequently. The samll capillary tube was condensed like a withered sprig (Fig. 4). In fibroma, the delicate reticular fibrils radiated from the capillary wall, presenting a close relation with the ribbon-like collagen fiber (Fig. 5).

4) *Simple impregnation of a paraffin section after Bielschowsky-Maresch (no use of potassium permanganate and oxalic acid).*

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Fig. 3. Fasciculated area of a neurinoma (Bielschowsky-Foot technique, paraffin section).

Fig. 4. Argyrophil fiber around the blood vessel in a nasopharyngeal fibroma (ibid.).

All the tissue element (nuclei, fibrils, and capillary wall) was impregnated rather non-specifically (Fig. 5).

5) *Impregnation of a frozen section with* 20% *silver bathe after Gros' modification.*

The Bielschowsky-Maresch method is not suitable for the demonstration of delicate peripheral neurofibrils generally. For the valuable modification of Stöhr-Seto, the fixation of neutral formalin is an absolute necessary condition. The writers obtained a fairy good result with the Goldin's modification (1937) of Bielschowsky-Gros technique. By this technique, the neurinoma fibril revealed a negative impregnation, while in the peripheral zone of the tumor, proliferating axis cylinder was seen (Fig. 7).

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Fig. 5. General view of a neurinoma (Bielschowsky-Maresch technique, paraffin section).

Fig. 6. Palisaded area of a neurinoma (Gros-Goldin technique, frozen section).

High power magnification revealed, in the interior, a delicate nodular fiber (seemingly corresponding to "Knötchenfaser Kimura") running through the tumor element rather sparely. All such element, however, should be regarded as a temporary phenomenon before ultimate degeneration of the nervous element. In fibroma, any notable change was not observed, perhaps owing to incomplete fixation.

Discussion.

On the view point of silver impregnation, Scherer (1934) pointed out that there is no specific mesenchymal fibril at present. From our observation too, the neurinoma is composed of a delicate argyrophil fibril, of a constant caliber and smooth impression, making a hair-like bundle, while the argyrophil capillary tube presents a striking contrast under certain condition. We are of opinion, therefore, that the neurinoma fibril is a product of the tumor cell, not of vascular origin, although the difference is purely morphologic. Again, it is not a true neurofibril. The contour of the tumor cell was not clear, while the fibril was included in the synplasmatic substance seemingly.

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Fig. 7. Neurofibrils in the peripheral zone of a neurinoma (Gros-Goldin technique, frozen section).

Fig. 8. Schematic representation of imaginated Schwannian tonofibrils and nuclear divisions on palisading.

According to Sekine (1943), "Neurilemmafaser" is nothing but a protoplasmic tube of the Schwannian cell impregnated by silver around the medullary sheath of the normal nerve. He regarded the neurinoma fibril as a product of neoplastic proliferation of those "Neurilemmafasern" roughly. Laidlaw (1930) demonstrated a double structure of the normal nerve sheath by silver impregnation, to which Zenker fixation was strongly

recommended. At any rate, such a tube structure of the normal nerve sheath was never proved on cross section of the fascicular bundle of a neurinoma. On the contrary, the so-called "Sonnenfigur", if occured ever, has been regarded a favorable finding to leiomyoma. The structural difference between a tube and a longitudinal fiber is, therefore, not unimportant problem on the morphology of a neurinoma. There is no explanation, why a tube-making substance in the normal state should be transformed into the delicate parallel fiber (neurinoma fibril), only when it concerns neoplastic proliferation.

Arai (1928) asserted on his experiment, transplantation of the peripheral nerve, that regenerating Schwannian cells change the mode of growth in accordance with their milieu. Only when the endoneurial sheath was preserved, he saw the regular course i.e. formation of "Kernstrangfaser" (Dürk) or "Bandfaser" (v. Büngner) corresponding to an elongated shape of the Schwannian cell. Thereby, it is evident that a certain supporting element should be necessary for a longitudinal arrangement of the Schwannian cell cord.

As is well-known, there are two types of neurinoma, fascicular (A-type of Antoni) and reticular (B-type of Antoni). In order to maintain a polarity of the Schwannian cell, protoplasmic streaming of the latter should occur longitudinally. There seems to be an intimate relation between fiber-producing effect and action current on the physico-chemical standpoint of view. The writers, however, supposed rather simply, that neoplastic Schwannian cells retain their fascicular shape by formation of tonofibrils as a metamorphosed basement membrane of the epithelial cell. Loss of polarity of the Schwannian cell will cause the B-type of Antoni, in which the longitudinal fibril is not present naturally. Partial condensation of the Schwannian cell-membrane into longitudinal fiber would not make a disturbance to a synplasmatic union of the neoplastic cells and to a parallel division of the palisading nuclei (Francini). As is shown above, neoplastic Schwannian cells in the fasciculated area do not present usually an individual cell territory. In parallel with the perpetual protoplasmic fusion, therefore, the newly formed fibril should slso be embedded into the synplasma.

Masson (1932) demonstrated, in his experimental schwannoma, an extracellular production of collagen by the proliferating schwannian cells. If the valuable data of Masson should be added to the writer's supposition, the neurinoma fibril is a condensed secretion of the Schwannian cell serving the purpose of a tonofibril (M. Haidenhain 1900), and of an external product principally. Perpetual fusion of the tumor cell would make an impression of intracellular fibrils apparently. Since the Schwannian cell, on its neoplastic change, was transformed into a fiber-producing cell, it should

be accepted that it concerns a degeneration of the noble element (Nageotte); a fibroblastic metaplasia in other words. In this connection, there is no sharp border line between the Schwannian cell and the endoneurial connective tissue cell. It is a biological feature of the peripheral glia to present an assimilating tendency in accordance with the milieu.

Masson called the fiber produced by Schwannian cell an ectodermal collagen. On advanced collagenization of a neurinoma, one may call it a neurofibroma. Writers believe that there exists a gradual transition between a neurinoma and a neurofibroma.

Thus, an ultimate end of malignant transformation of a neurinoma, if it occurred ever, would result in a neurofibrosarcoma or a neurosarcoma. Mixed proliferation of Remak's fiber was described occasionally, although it would not be a true nerve fiber, but merely an imitation product by the neoplastic cell, presenting an analogous cell-figure to the fibocytes.

It is generally accepted to classify a tumor according to its genetic origin. Writers lay much stress upon th present condition, i.e. carcinoma or sarcoma should be diagonsed by the present state of an epithelial or a non-epithelial feature solely. If the embryological systematization should be hold strictly, a malignant hypernephroma or an endothelioma of the serous cavity will be classed into a mesodermal carcinoma. Thus, there is no reason to suppose that the neuroectodermal cell never changes into a true sarcoma.

At present, nerve sheath tumor or neurilemmoma proposed by Stout is more inclusive than neurinoma. If the ectodermal fibroblast should be accepted, perineurial fibroblastoma proposed by Mallory and Penfield might have been not so inadequate term, although it is not advantageous to be confused with the proper fibroblast, i.e. a wandering cell of the granulation tissue.

Masson compared the palisaded structure of a neurinoma with the Meissnerian body of the tactile nerve ending. From a standpoint that the Schwannian cell is a peripheral glia, the neurinoma fibril and the naevocellular argyrophilic fiber belong to a peripheral neuroglia fibril. In other words, the argyrophilic fiber includes a peripheral glia. Even though the Schwannian cell is the homologon of the oligodendroglia of the central nervous system, there is a marked difference between the two elements morphologically. Non-utility of the specific impregnation methods of the central glia to the neoplastic Schwannian cell (Tarlov) does not afford a favorable condition to the mesodermal origin of neurilemmoma. Under certain condition, Schwannian cell cord was demonstrated by Hortega-Penfield's technique for the central glia (Kurobane).

It may be supposed reasonably that there are many sorts of argyrophil fiber in the human body, but it is a question whether any silhouette

image can be judged properly, disregarding the milieu and the background. The artificial reticulum demonstrated by Nageotte and Guyon (1930) is lacking in cellular elements and vascular formation, whatever it presents a striking resemblance to the vital reticulum of the human body. The writers intend to give attention to the Reinhart's paper (1930) forgotten by many investigators.

Conclusion.

1) The neurinoma fibril was impregnated most clearly by Bielscowsky-Maresch's method on a frozen section.

2) Bleaching plan by potassium permanganate and oxalic acid is useful for differentiation of the neurinoma fibril from the reticulum fiber of vascular origin.

3) The neurinoma fibril is regarded as a tonofibril produced by the neoplastic Schwannian cell.

4) A fibroblastic metaplasia of the neoplastic Schwannian cell was discussed.

5) On the viewpoint of peripheral glia, the neurinoma fibril belongs to a peripheral neuroglia fibril.

6) All the argyrophil fiber is not a unitary entity. On the morphologic standpoint, it would be necessary to recognize the special character of fibers, according to their milieu.

References.

1) Scherer, Virchows Arch., 1934, **292**, 251.
2) Sekine (in Japanese), Gann, 1943, **37**, 147.
3) Laidlaw, Am. J. Path., 1930, **6**, 435.
4) Arai, Mitt. path. Anat, zu Sendai, 1928, **4**, 345.
5) Sekiguchi and Oye, ibid., 1928, **4**, 51.
6) Masson, Am. J. Path., 1932, **8**, 367.
7) Endo (in Japanese), Tohoku Igaku Zasshi, 1933, **16**, 205.
8) Stout, Am. J. Cancer, 1935, **24**, 751.
9) Mallory and Parker, Am. J. Path., 1927, **3**, 515.
10) Tarlov, Am. J. Path., 1940, **16**, 33.
11) Hasegawa, Oyama, Kurobane and Sasano, Fol. psych. Jap., 1949, **3**, 234.
12) Nageotte and Guyon, Am. J. Path., 1930, **6**, 631.
13) Reinhart, ibid., 1930, **6**, 535.
14) Willis, Pathology of Tumours., London, 1948.
15) Romeis, Taschenbuch der mikroskopischen Technik, 13. ed., Berlin 1932.
16) Ramon-Cajal, Histology. William Wood & Company, Baltimore, 1933.

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On the Cobalt Polycythemia.

By

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Introduction.

In 1929, Waltner and Waltner[1] discovered that polycythemias could be produced experimentally in rats by feeding cobalt. Since then many reports[2] were published on this subject, and it became clear that the experimental cobalt polycythemias could be induced also in rabbits (Kleinberg)[3] as well as in dogs (Barron and Barron[4]). In considering the hematopoietic substances of which iron is the representative, it is well known that all of them merely hasten the recovery from anemia when it occurs, and never cause polycythemia when administered to normal animals. On the contrary, cobalt finds its way into animals in normal condition and brings about polycythemia in them.

Generally speaking, constancy of erythrocyte count in the blood is maintained by a balance of two processes, that is a process of being constantly produced on the one hand and a process of being destroyed on the other. Polycythemia is a condition brought about by a disturbance in the equilibrium of these two processes. According to Orten,[5] the state of experimental polycythemia by cobalt is a result of increased production of erythrocytes. While the experimental polycythemia by cobalt has been known to be inhibited by various substances, e.g., by vitamine C (Barron and Barron,[4] Davis[6]), choline (Davis[7]) and histidine and cystein (Orten[8]), the fundamental mechanism at work in cobalt polycythemia is still unknown. Recently vitamine B_{12}[9][10], one of the most outstanding of substances having hematopoietic actions, has been found to be a compound of cobalt; a fact very interesting as well as significant, indeed.

The present report concerns my experiments performed on rabbits to elucidate more closely the features of the cobalt polycythemia.

Experimental.

Methods.

Eleven rabbits were used in the experiments. The cobalt was given

in form of cobalt sulfate. The dose was 4 mg. per kilogram of body weight, per day. For counting red cells, the blood from the ear vein was used. Dilution of blood and enumeration of cells were carried out as usual. Reticulocyte count was performed by a supravital technique with brilliant cresyl blue and its result was expressed in ‰ of all red cells. Blood hemoglobin content (in terms of oxygen capacity, vol. %) was measured by the acid hematin colorimetric method of Cohn and Smith.[13] As the erythrocyte count and hemoglobin content of the rabbit's blood varies considerably with the season, it is not easy to determine the color index. Therefore, the time of least seasonal variation was selected for experiments and the mean corpuscular hemoglobin before the institution of cobalt administration was taken as standard, that is 1.0 in color index. Leukocyte was counted by an ordinary method after diluting blood in Türk's solution.

Results.

Course of cobalt polycythemia. Intravenous injection of cobalt sulfate in the above-mentioned dose was carried on for 14 days. The course of polycythemia then is shown in Fig. 1. Following the start of injection, first of all, reticulocyte began to increase after a latency of two to four days. The onset of its increase was usually outstanding and the increase kept on

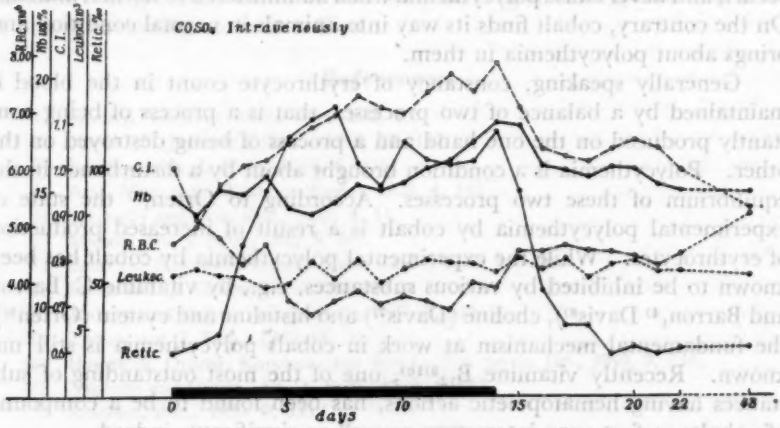

Fig. 1. Effect of the intravenous injection of cobalt sulfate on the blood picture in rabbit. Black bar below indicates the period of injection.

going while the injection continued. About one week after the start, the reticulocyte count attained its maximum, remained thereafter about the same though with some fluctuations. Erythrocyte began to increase somewhat later than the onset of reticulocyte increase, followed by an in-

crease of hemoglobin content. In contrast to these, the color index decreased steadily until it reached a difinite value considerably lower than normal; this level was kept constant throughout the period during which the injection was continued.

When the injection was discontinued reticulocyte count returned, in two to four days, to the value before the injection, along with the return of the erythrocyte count and hemoglobin content to their original levels. The color index also returned to 1.0; sometimes it became temporarily over 1.0. No definite effect on the leukocyte count was observed all through the course. Normoblasts did not appear in smears even when polycythemia was most remarkable.

By subcutaneous injection, as it is shown in Fig. 2, the effects were about the same as those by intravenous administration except that the course in the former case was not so acute as in the latter.

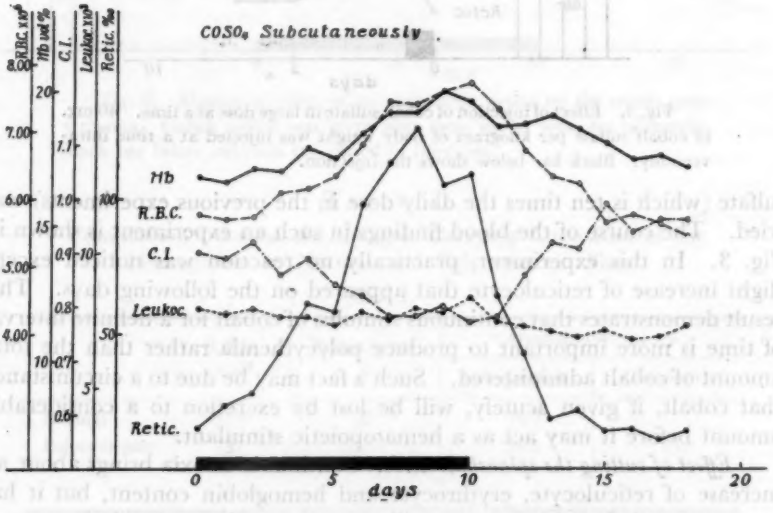

Fig. 2. Effect of the subcutaneous injection of cobalt sulfate on the blood findings in rabbit. Black bar below also indicates the period of administration.

Effect of injection of cobalt in large dose. In the above-mentioned experiments small dose of cobalt was given daily but for elucidating the mechanism of polycythemia brought about by cobalt, it is of no little significance to inquire whether the total amount of cobalt given in the above experiments, when it is administered all at a time, might also produce polycythemia or not. An intravenous injection of 40 mg. dose of cobalt

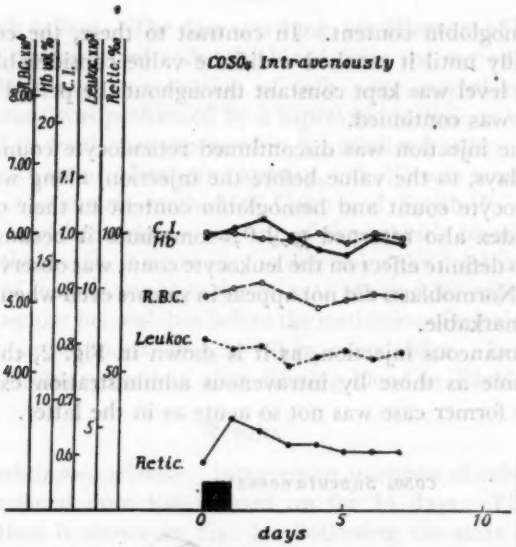

Fig. 3. Effect of injection of cobalt sulfate in large dose at a time. 40 mg. of cobalt sulfate per kilogram of body weight was injected at a time intravenously. Black bar below shows the injection.

sulfate (which is ten times the daily dose in the previous experiments) was tried. The course of the blood findings in such an experiment is shown in Fig. 3. In this experiment, practically no reaction was noticed except slight increase of reticulocyte that appeared on the following days. This result demonstrates that continuous stimulus of cobalt for a definite interval of time is more important to produce polycythemia rather than the total amount of cobalt administered. Such a fact may be due to a circumstance that cobalt, if given acutely, will be lost by excretion to a considerable amount before it may act as a hematopoietic stimulant.

Effect of cutting the splanchnic nerve. Continuous anoxia brings about an increase of reticulocyte, erythrocyte and hemoglobin content, but it has been reported that this increase will not appear when splanchnic nerve is cut.[12] To know whether or not polycythemia by cobalt has any relation to the splanchnic nerve the following experiment was performed. First the nerve were cut bilaterally, and subsequently cobalt salt was injected. The course thereof is shown in Fig. 4. As shown in the figure, nothing particular in the course of polycythemia was observed. Thus it is clearly established that the splanchnic nerve has no relation to this type of experimental polycythemia.

Oxygen capacity of blood hemoglobin which increased by cobalt. In the experiments reported above, the increase of blood hemoglobin by cobalt was

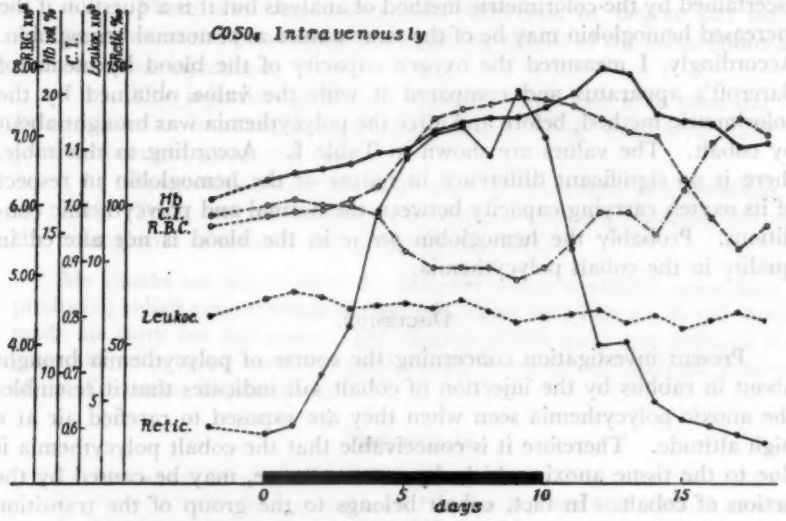

Fig. 4. Absence of effect of the splanchnic nerves on the cobalt polycythemia. Cobalt sulfate was given after these nerves were cut bilaterally. Black bar below indicates the period of injection.

TABLE I.

Comparison between the Oxygen Carrying Capacities of the Hemoglobin in Normal and Polycythemic Blood.

Case 1.

	R.B.C. $\times 10^6$	Reticulocyte ‰	Hemoglobin vol. %	
			Colorimetric	Gasometric
Normal	4.95	28	14.7	
Polycythemic	7.12	125	20.0	19.2

Case 2.

	R.B.C. $\times 10^6$	Reticulocyte ‰	Hemoglobin vol. %	
			Colorimetric	Gasometric
Normal	4.45	30	14.8	
Polycythemic	7.03	98	19.2	19.4

Experimental animal: rabbit. Cobalt sulfate was injected 4 mg. (in Case 1) or 8 mg (in Case 2) per kilogram of body weight per day, intravenously. After 15 days (in Case 1) or 19 days (in Case 2), when polycythemia reached its maximum, the oxygen carrying capacity of blood was measured by means of Barcroft's apparatus and compared it with the value obtained by colorimetric method. There is no significant difference between the two values.

ascertained by the colorimetric method of analysis but it is a question if the increased hemoglobin may be of the same nature as of normal hemoglobin. Accordingly, I measured the oxygen capacity of the blood by means of Barcroft's apparatus and compared it with the value obtained by the colorimetric method, before and after the polycythemia was brought about by cobalt. The values are shown in Table I. According to this table, there is no significant difference in nature of the hemoglobin in respect of its oxyten carrying capacity between the normal and polycythemic conditions. Probably the hemoglobin *per se* in the blood is not altered in quality in the cobalt polycythemia.

Discussion.

Present investigation concerning the course of polycythemia brought about in rabbits by the injection of cobalt salt indicates that it resembles the anoxic polycythemia seen when they are exposed to rarefied air at a high altitude. Therefore it is conceivable that the cobalt polycythemia is due to the tissue anoxia which, by some measure, may be caused by the action of cobalt. In fact, cobalt belongs to the group of the transition elements as iron does, and iron plays an important part in the tissue oxidation as an indispensable component in iron porphyrin compounds. It may be assumed that cobalt inhibits the biological oxidation. It is a difficult problem, however, to elucidate what part cobalt plays in the biological oxidation, a problem to be solved hereafter.

It was ascertained, at least, that as to the oxygen capacity, the increased hemoglobin in the cobalt polycythemia is of the same nature as the normal hemoglobin. This is quite in accord with a recent report of Orten[11] on cobalt polycythemia in rats. Hence it is unlikely that cobalt polycythemia is due to general *anoxemia*. Of course, the anoxia in the tissue of bone marrow can not be denied.

Concerning a question whether or not the splanchnic nerve plays parts in the cobalt polycythemia, the result was negative in my experiment, notwithstanding the fact that the nerve was said to have some influence on the polycythemia produced by lowered barometric pressures.

Finally, one injection of cobalt in a large dose does not bring about any significant hematological change. This is presumably ascribed to the fact that cobalt injected will be excreted in a comparatively short time.

Summary.

(1) It was confirmed that the successive injection of cobalt into rabbits day after day brings about a definite increase of reticulocyte, followed by an increase of erythrocyte and hemoglobin content. There is no change in leukocyte count.

(2) There was observed a little difference in degree but not in nature between the polycythemia producing effects of the intravenous and subcutaneous injections.

(3) The injection of a large dose of cobalt salt at a time did not bring about an increase in erythrocyte count. Only successive, daily injections brought about the effect.

(4) Splanchnic nerve plays no part in the cobalt polycythemia.

(5) There was no difference in oxygen carrying capacity between the hemoglobin of normal and polycythemic blood.

My thanks are due to Prof. K. Matsuda whose successful experiment in producing cobalt polycythemia in dogs and whose keen interest to this subject made me carry out this study. The expense of this study was defrayed by a grant offered to him from the Foundation for Promotion of Scientific Research of the Education Department.

References.

1) Waltner and Waltner, klin. Wschr. 1929, **8**, 313.
2) Orten, Underhill, Mugrage and Lewis, Proc. Soc. Exp. Biol. Med., 1931–32, **29**, 174.
3) Kleinberg, Amer. J. Physiol., 1934, **108**, 545.
4) Barron and Barron, Proc. Exp. Biol. Med. 1935–36, **35**, 407.
5) Orten, Amer. J. Physiol., 1935, **114**, 414.
6) Davis, Amer. J. Physiol., 1940, **129**, 140.
7) Davis, Amer. J. Physiol., 1940, **129**, 129, P 344.
8) Orten and Bucciero, J. Biol. Chem. 1948, **176**, 961.
9) Rickes, Brink, Koniuszy, Wood and Folkers, Science, 1948, **108**, 134.
10) Smith, Nature, 1948, **161**, 638.
11) Bucciero and Orten, Blood, 1949, **4**, 395.
12) Ueda, Kaigungunikaizasshi, (Bull. Naval Med. Ass. Japan), 1944, **33**, 34.
13) Peters and van Slyke, Quantitative clinical chemistry, Vol. 2, Methods, The Williams and Wilkins Co. Baltimore, 1932, p. 668.

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Hematopoietic Actions of Some Complex Salts of Cobalt.

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(Received for publication, April 21, 1950)

Introduction.

Since the discovery of cobalt polycythemia by Waltner and Waltner[1] in 1929, Orten and others[2] have carried out numerous observations about this phenomenon and all of them never failed to confirm the polycythemia producing actions of cobalt in animals. They administered cobalt to experimental animals mostly in form of its salts such as chloride, sulfate or nitrate. Chemically, it is well known that cobalt often forms several complex salts as iron does, and hematopoietic actions of such complex salts of cobalt have not yet investigated so far. The complex salts deserve of our special attention since hemoglobin and other important enzymes which are indispensable in the intracellular oxidations contain iron in form of complex salt as one of the most essential components in its chemical structures. Moreover, Tsumaki et al.[3] reported recently that a cobalt complex salt synthetized by them was found to combine with oxygen in the same manner as hemoglobin. Taking these facts into consideration, it seems interesting to investigate whether complex salts of cobalt have hematopoetic action as ordinary cobalt salts or not, and when they have, to investigate whether their actions be of any particular nature or not. The present report deals with such an investigation.

Experimental.

Methods.

The experiments were to test hematopoietic actions of various complex salts of cobalt on rabbits. Cobalt complex salts used here are as follows: *Purpureo salt* ($[Co(NH_3)_5Cl]Cl_2$), *carbonatotetrammine cobaltic nitrate* ($[Co(NH_3)_4CO_3]NO_3$), *Flavo salt* ($[Co(NH_3)_4(NO_2)_2]Cl$), and *Croceo alt* ($[Co(NH_3)_4(NO_2)_2]Cl$). *Purpureo salt* is a pentammine cobaltic salt and

others are tetrammine cobaltic salts. *Flavo salt* and *Croceo salt* are isomers, that is, in *Flavo salt*, two nitroradicals take the *cis* position and in *Croceo salt*, the *trans* position.

All these cobalt complex salts were solved in distilled water and injected intravenously. The dose was 4 mg. per kilogram of body weight per day. This is a sufficient dose as cobalt to produce polycythemia in rabbits as reported in my previous communication.[4] Techniques for the enumeration of erythrocyte and reticulocyte and for the determination of hemoglobin concentration in blood were the same as in my previous report.

Results.

Purpureo salt: The course of the effect of this aslt is shown in Fig. 1. As observed in this figure, this complex salt had no effect on the hematopoietic organs except of slight increase in reticulocyte count. Color index remained normal throughout. There was no change also in leukocyte count.

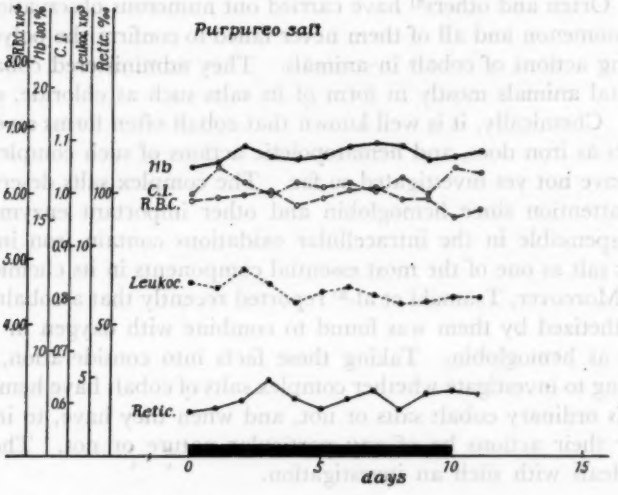

Fig. 1. Effect of the administration of *Purpureo* salt on the blood picture in rabbit. Black bar below indicates the period of injection.

Carbonatotetrammine cobaltic nitrate: Fig. 2 shows the course of blood picture when *carbonatotetrammine cobaltic nitrate* was given. Contrary to *Purpureo salt*, this complex salt produced distinct polycythemia; the course of change in erythrocyte, reticulocyte counts and in hemoglobin concentration was similar to that observed in the polycythemia by cobalt sulfate, though the effect of this complex salt was weaker than that of ordinary

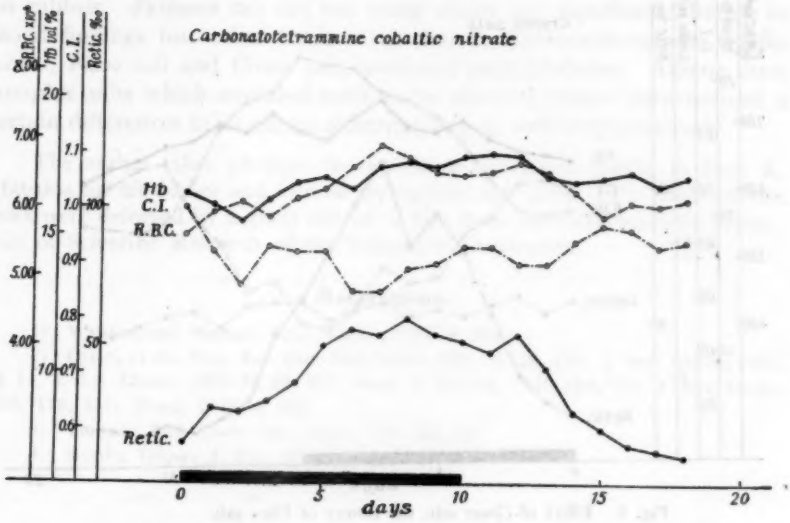

Fig. 2. Effect of the *Carbonatotetrammine cobaltic nitrate*, the pentammine cobaltic salt.

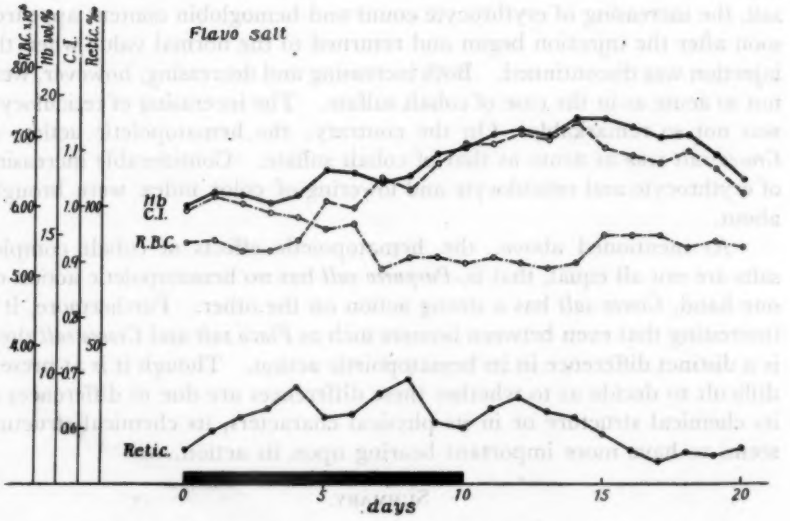

Fig. 3. Effect of the *Flavo* salt, the isomer of *Croceo* salt.

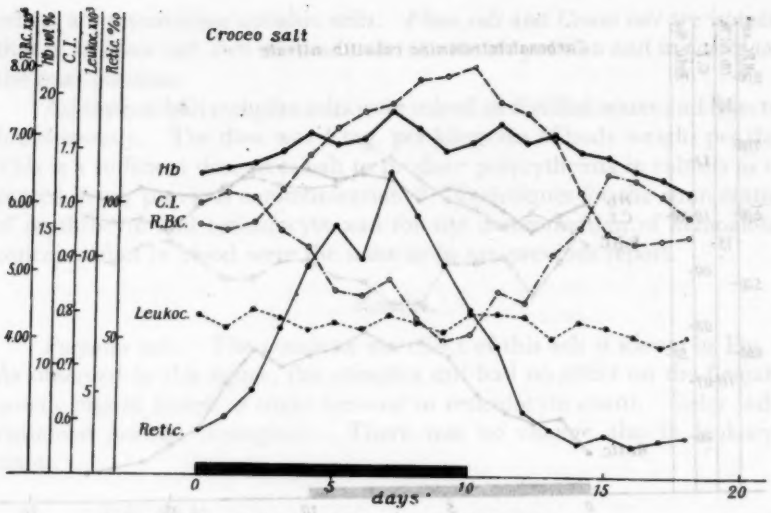

Fig. 4. Effect of *Croceo* salt, the isomer of *Flavo* salt.

cobalt salts. Color index steadily decreased during injection and returned to normal value in four days after the injection had been stopped.

Flavo salt and *Croceo salt:* Fig. 3 shows the effect of *Flavo salt*, and Fig. 4 of *Croceo salt*. These are isomers as mentioned above. By *Flavo salt*, the increasing of erythrocyte count and hemoglobin content appeared soon after the injection began and returned to the normal value when the injection was discontinued. Both increasing and decreasing, however, were not so acute as in the case of cobalt sulfate. The incerasing of reticulocyte was not so remarkable. On the contrary, the hematopoietic action of *Croceo salt* was as acute as that of cobalt sulfate. Considerable increasing of erythrocyte and reticulocyte and lowering of color index were brought about.

As mentioned above, the hematopoietic effects of cobalt complex salts are not all equal, that is, *Purpureo salt* has no hematopoietic action on one hand, *Croceo salt* has a strong action on the other. Furthermore, it is interesting that even between isomers such as *Flavo salt* and *Croceo salt* there is a distinct difference in its hematopoietic action. Though it is at present difficult to decide as to whether these differences are due to differences in its chemical structure or in its physical characters, its chemical structure seems to have more important bearing upon its action.

Summary.

The hematopoietic actions of four complex salts of cobalt were examined

on rabbits. *Purpureo salt* did not bring about any significant altering in blood findings but other three salts, that is, *Carbonatotetrammine cobaltic nitrate*, *Flavo salt* and *Croceo salt*, produced polycythemias. Among these complex salts which revealed itself to be effective, there were noticed a certain differences in its effects quantitatively as well as qualitatively.

The auther takes pleasure in expressing his sincere thanks to Prof. K. Matsuda for his advice and interest throughout the work. The costs of this work were defrayed by a grant offered to him from the Foundation for Promotion of Scientific Research of the Education Department.

References.

1) Waltner, and Waltner, Klin. Wschr., 1929, **8**, 313.
2) Orten, et al., Proc. Soc. Exp. Biol. Med., 1931-32, **29**, 174; J. Biol. Chem., 1932, **96**, 11, J. Biol. Chem., 1932-33, **99**, 457; Amer. J. Physiol., 1935, **114**, 414; J. Biol. Chem. 1948, **176**, 961; Blood, 1949, **4**, 395.
3) Tsumaki, Bull. Chem. Soc. Japan, 1938, **13**, 252.
4) Suzuki, Tohoku J. Exp. Med., 1951, **53**, 359.

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ABO-Blood-Group Lipoids in Human Liver.

Second Report.*

By

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(Received for publication, May 5, 1950)

Group A lipoid from human liver (prepared as reported[1]) was hydrolyzed at first with dilute H_2SO_4 and at intermediate stages with alcoholic H_2SO_4-, watery HCl-, alcoholic $Ba(OH)_2$- and watery NaOH-solution with fractionation and glucosamine, galactose, choline, arginine and palmitic acid were separated and identified. Besides a high-molecular unsaturated hydroxyacid, a probable ketoalcohol and three substances containing N and P were obtained. The consequences of qualitative tests suggested also the presence of traces of formic acid, tyrosine, xanthine and hypoxanthine or guanine among the hydrolysis products. Not only did the group-lipoid not give the acrolein reaction[1] but also among its cleavage products glycerol as well as glycerophosphoric acid was not found. Thus the possibility that the material was contaminated with lecithin, cephalin or plasmalogen was excluded. In Table I, yields of the separated substances are listed. Those components which were so scarce as were detected only by qualitative tests are assumed naturally as impurities, although those separated can not be necessarily accepted all as proper constituents.

The crop of glucosamine (isolated as hydrochloride) was less than equimolecular to that of galactose. But, for the reason mentioned in the preceding report[1], i.e., acid hydrolysis of the lipoid gave a water-insoluble, acid-groups-possessing substance on which a part of glucosamine possibly adsorbed, as many molecules of the hexosamine are porbably involved as those of galactose in this group substance. Masamune[2] postulated from the positive Pettenkofer reaction (an assay with saccharose and conc. H_2SO_4)[3] that in group lipoids sphingosine takes the place of hexosamine in group carbohydrates, whereas the writer definitely proved the absence of sphingosine by the present investigation (s. Exp. 17), simultaneously

* 5th report of Masamune and co-workers' "Chemistry and Biology of Lipids."

TABLE I.

The Yields of Separated Substances after Hydrolysis of 3.1 g. Group A Lipoid from Human Liver.
The lipoid contained 5.7% ash.

Substance	Separated derivative and weight of crude product	Yield of the substances			
		mg.	% of the lipoid		Proportional number of molecules
			Found	On ash-free basis	
Galactose	Methyphenylhydrazone, 133 mg.	84.2	2.7	2.9	1.0
Glucosamine	Hydrochloride, 69 mg.	57.3	1.9	2.0	0.71
Arginine	Flavianate, 43 mg.	15.3	0.49	0.52	0.19
Choline	Chloroplatinate, 43 mg.	14.5	0.47	0.50	0.26
Palmitic acid	Separated as such	918 ($850\times\frac{27}{25}$)	29.6	31.4	7.7
An unsaturated hydroxy acid	" " "	540	17.4	18.4	1.2
A probable keto-alcohol	" " "	100			
N.P.F. I	" " "	100			
N.P.F. II	" " "	350			
N.P.F. III	" " "	200			

finding that that reaction is given even by palmitic acid. Accordingly not psychosin but glucosamin-egalactoside acts as a determinant group in blood group substances of lipoid nature like in those of carbohydrate nature.

Whether all or a few of the constituents of the group lipoid participate in the group reaction and how the constituents are linked together will be settled by a further study now in progress.

EXPERIMENTAL.

The Group A lipoid was prepared as described in the first paper.[1] The product resembled those in that account. N 1.90%, P 4.5%, glucosamine 2.4%, galactose 2.6%, ash 5.7%.

1. *Hydrolysis of the Group Substance.* 3.1 g. of the material were finely dispersed in 180 cc. of 1 n. H_2SO_4 and refluxed on a boiling water-bath for 5 hours, bubbling with CO_2 gas, and cooled in ice, whereby dark brown oil that had appeared during the hydrolysis changed solid. This was filtered off and washed with water (20 cc.).

2. *Fractionation of the Water-soluble Substance.* The brown filtrate and

washing were combined and exhausted with ether, after which 20% phosphotungstic acid was added until no more precipitation occurred. Stood for 1 day. The precipitates were centrifuged, washed with 5% H_2SO_4 containing 2.5% phosphotungstic acid.

The ether extracts (light yellow) in the above were evaporated to about 3 cc. To the condensate (pH 2.0, brown) was added 15 cc. water and a small amount of oil separating thereby was removed. The solution (light yellow) reduced Tollens reagent and gave mercuric chloride test for formic acid. It changed dark brown on alkalizing to pH 10. After shaking with changes of ether, it was again acidified (to pH 1.0) and, treated with ether. The extracts were condensed to about 2 cc., mixed with 10 cc. of 3% H_2SO_4 and after addition of 2 g. Na_2SO_4, subjected to steam distillation. The distillate (Tollens +) gave no precipitates with uranylformiate and sodium formiate (acetic acid —).

3. *Examination and Fractionation of the Substance Non-precipitable by Phosphotungstic Acid.* The mother liquid of the phosphotungstic acid-precipitates and washing of the latter were exhausted with ether and $Ba(OH)_2$ added under agitation until pH of the solution was raised to 6.2. The precipitates were centrifuged and washed with boiling water again and again with centrifugation, until no more Molisch-giving substance came out, and all the supernatant liquids were distilled together to 100 cc. pH of the condensate lay at 4.2. Hereupon 25 cc. of 25% mercuric acetate solution were added and gray precipitates separated and washed with 10 cc. of 5% mercuric acetate solution (pH 4.2). To the mother liquid and the washing of the precipitates were added the saturated $Ba(OH)_2$ to pH of 7.0, whereby yellowish precipitates were given. Centrifuged. From the centrifugate was isolated glucosamine as hydrochloride and from the supernatant liquid galactose as methylphenylhydrazone as will be presently described.

4. *Isolation of Glucosamine*[4] *as Hydrochloride.* The final centrifugate was suspended in water (150 cc.) and freed from barium with H_2SO_4 (H_2SO_4 was added to pH 2.0 of the solution) and from mercury with H_2S. The solution obtained was distilled in vacuo to reduce the volume to two thirds (pH was lowered to 1.2) and after passing ether (for removal of acetic acid) and addition of 2 cc. of conc. HCl, just sufficient baryta added to precipitate out excess H_2SO_4. The centrifuged supernatant liquid and washings of the centrifugate here were combined, condensed to about 5 cc., and 2 cc. of conc. HCl and 30 cc. of alcohol added. The mixture was stood in an ice chest for 2 days. The crystalline deposits were filtered on a suction funnel (69 mg. corresponding as glucosamine to 2.0% of the lipoid), redissolved in 2 cc. of water and recrystallized by addition of 0.5 cc. of conc. HCl, 5 cc. of alcohol and 35 cc. of acetone and standing. Tetra-

gonal plates were given. Yield, 53 mg. The product began to soften at 180° and to darken at 200° and charred at 240°C. Fehling (hot) and Tollens (reduced very slowly) solution were reduced. Colour development with 0.802 mg. of it by Hamasato-Akakura method (Heating with 5.5 n. HCl containing $SnCl_2$ was omitted) corresponded in intensity to 0.80 mg. glucosamine hydrochloride. N (micro Kjeldahl), 6.41% (calc., 6.48%). $[\alpha]_D^{10}$ (in water) was $+98.5° \left[=\frac{2.05\times 1}{0.0208\times 1}\right]$ after 5 minutes from dissolution, which changed to the equilibrium value of $+72.6° \left[=\frac{1.51\times 1}{0.0208\times 1}\right]$ in 8 hours.

5. *Isolation of Galactose.* The mother liquid of mercuric oxide with which glucosamine precipitated together (s. 3.) was removed from barium and mercury and exhausted with ether similar to above (Conc. HCl was not employed). After vacuum distillation to about 3 cc., it was mixed with 3 cc. alcohol, 0.18 cc. 50% acetic acid and 0.15 g. α-methylphenylhydrazine and stood at 37°C for 6 hours and at 0°C overnight. The crystals (narrow plates) separated amounted to 133 mg. (as galactose 84.2 mg. or 2.7% of the lipoid). The product was purified repeatedly from 40 cc. of hot 90% alcohol to a constant melting point of 189°C. Mixed melting point test with galactose-methylphenylhydrazone showed no depression. Soluble in pyridine and hot ethanol but insoluble in water. N (Friedrich) 9.61% (calc., 9.86%). It had no rotation in pyridine at all (measured on a 3% solution at 16.3°C).

6. *Examination of the Substance Precipitated at pH 4.2 with Mercuric Acetate.* The precipitates were suspended in 20 cc. of a very dilute H_2SO_4 (pH 2.0) and H_2S gas passed. Mercuric sulfide was centrifuged, washed and then centrifuged off. The non-colored supernatant liquids were united, nearly neutralized with baryta (The precipitates rejected) and distilled in vacuo to about 3 cc. The condensate gave the positive test for amino acids (ninhydrin test) and also those, though faint, for tyrosine (an improvement of Millon test, xanthoprotein test) and arginine (Sakaguchi). The reactions of tyrptophane (Cole) and histidine (Totani, Hanke-Koessler) did not occur.

7. *Fractionation of the Substance Precipitated with Phosphotungstic Acid.* The precipitates by phosphotungstic acid, centrifuged and washed, were suspended in 30 cc. of a dilute H_2SO_4 (pH below 1.0) and shaken with changes of ether in a separatory funnel. Black tarry matter separated and floated between the watery and ethereal layer. It was collected, dissolved in dilute alkali and precipitated with acid. The precipitates were washed with alcohol and dried—**N.P.F. I.** The amount was about 100 mg. It contained nitrogen (1.1%) and phosphorus (qualitatively tested) but

gave neither the saccharose-conc. H_2SO_4 reaction of sphingosine nor acrolein reaction of glycerol. Ash 56.5%. Further study was not made.

The yellowish watery layer was freed from sulfuric acid with baryta (The solution after this treatment was at a pH of 6.5) and distilled in vacuo to a small volume (about 1 cc.), whereupon crystals in plates and in irregular form and amorphous precipitates appeared. Water was added up to some 10 cc. to redissolve the deposits. Tests (A small part of the solution was applied): a) alloxan test for choline resulted in positive and addition of alcoholic platinum chloride solution gave precipitates, b) the positive reaction of arginine was given, c) picrolonic (in water) and picric (in water and in alcohol) acid, sodium picrate (in water) and gold chloride (in water) gave rise to no precipitation.

Then the solution was acidulated with dilute HNO_3 and just sufficient $AgNO_3$ (20% solution) added to cast down all the precipitable substance. Centrifuged. The centrifugate was decomposed, after suspending in 15 cc. of water, with a smallest possible quantity of HCl (10%). The centrifuged supernatant liquid here was distilled under diminished pressure with alcohol many times. And the last residue (a small quantity) was taken up in 0.5 cc. water for qualitative assays. A very small insoluble part was centrifuged off. The clear supernatant liquid (pH 4.5) gave diazo (with NaOH) and murexide (with halogen) reaction. Thus adenine did look to be absent because of no occurrence of precipitation by picric acid (the result of test on the solution before fractioning with $AgNO_3$), whereas the presence of xanthine (because of the positive murexide reaction) and hypoxanthine or guanine (A substance precipitable with $AgNO_3$ but insoluble in water was present, and on the other hand neutralization with NH_3 of the centrifuged supernatant liquid gave no precipitation) were suspected.

The mother liquid of the $AgNO_3$-precipitates was treated with H_2S and the centrifuged supernatant liquid was liberated from excess HNO_3 by repeated vacuum distillation with water and with alcohol. From the residual arginine and choline were separated as follows.

8. *Isolation of Choline.* The crystalline mixture free from purines was agitated with 30 cc. of absolute alcohol and centrifuged. The supernatant liquid was condensed to about 0.5 cc. and sufficient alcoholic platinum chloride solution (2%) added. Brown amorphous deposits were separated and after drying, dissolved in 2 cc. of water and evaporated on a boiling water-bath, whereby the substance crystallized in rhombic form. 43 mg. F.P. 233°C. N(Friedrich) 4.47% (calc. for choline chloroplatinate $(C_5H_{14}ONCl)_2PtCl_4$, 4.54%). Long dark brown, dichroistic and double-refractive crystals were given by iodine potassium iodate (Rosenheim).

9. *Isolation of Arginine.* The alcohol-insoluble part, which was

free from choline and biuret positive, was dissolved in 50 cc. of water and barium precipitated by the aid of an equivalent quantity of H_2SO_4. The mother liquid was distilled to 5 cc., 5 cc. of 12 n. HCl were added and hydrolysis processed by heating under reflux for 16 hours in boiling water. The brown hydrolysate was diluted with water, the humin filtered off and the filtrate distilled to dryness. The remainder was further distilled either with alcohol or with water again and again to eliminate HCl as far as possible. Finally it was taken up in 2 cc. of water (pH of the solution was 4.0) and after addition of 5 cc. of 5% flavianic acid (solvent, water), stood for 1 day. Yellow crystalline deposits were centrifuged and recrystallized from 10 cc. of a hot, very dilute flavianic acid solution. The substance (microscopic octahedrons) was dried at 105°C. Yield, 43 mg. It began browning at 258°C and decomposed at 268°C. N (Friedrich) 17.15% (calc. for arginine flavianate $C_6H_{14}O_2N_4 \cdot C_{10}H_6O_8N_2S$, 17.21%).

10. *Fractionation of the Matter that Precipitated out during the Primary Hydrolysis of the Group Lipoid.* The substance was colored dark brown and weighed 2.7 g. 2.5 g. of it were stirred up in 500 cc. of 0.1 n. NaOH. The gray insoluble part was centrifuged and washed with 10 cc. of the alkali. The dark brown supernatant liquid and washing were acidified to pH 1.0 with H_2SO_4 and shaken with changes of ether. The watery layer became almost colorless and a dark brown tarry substance soluble neither in the dilute acid nor in ether appeared which was separated. On evaporation of the ether extracts there remained dark brown substance. This weighed 1.87 g.

11. *Examination of the Substance Remaining Undissolved in Dilute Alkali* (s. the preceding section). This fraction (grayish waxy substance, 100 mg.) was found soluble in chloroform and benzene but insoluble in water, dilute acid, alcohol, acetone and ether. In hot dilute alkali it dissolved and came out on cooling in the form of jelly. For the assays below a small chloroform-insoluble part was removed. When heated in a capillary tube the substance fused mostly at 75°C into brown oil and, turning black at about 200°C, solidified at about 230°C. On burning it gave practically no ash. Neither nitrogen (Lassaigne) nor phosphorus (ammonium molybdate test with oxidation) was detected, offering an evidence in disproof of its being sphingosine. Violet colour developed when it was stood with saccharose and conc. H_2SO_4[3]. Fehling, Molisch and Liebermann-Burchard (for cholesterol) reaction were not given, but tests for alcohol (with diazobenzenesulfonic acid and with vanilline) and ketone (Piloty-Stock) resulted in positive. Thus it appeared to be a ketoalcohol, although the possibility is not excluded that it was a high-molecular acid because of its solubleness in hot alkali mentioned.

12. *Examination of the Substance in* 10 *which Dissolved in Dilute Alkali*

but did not in Dilute Acid and Ether. It was dissolved in 30 cc. of 0.1 n. NaOH and precipitated by addition of H_2SO_4 to pH 1.0 of the solution. The centrifugate was washed with water and dried.—**N.P.F. II.** About 350 mg. were given. Soluble in alcohol and acetone. Insoluble in water, dilute H_2SO_4, ether, benzene, chloroform and petroleum ether. N (micro Kjeldahl), 1.5%. P (modified Plimmer method), 4.3%. Ash, 0.3%. It did not give violet color with saccharose and conc. H_2SO_4 (Thudicum test for sphingosine) and acrolein reaction of glycerol.

Saponification of the substance was tried as follows:— 250 mg. of it were suspended in 20 cc. methanol containing 2% $Ba(OH)_2$, and boiled under reflux for 9 hours but almost no dissolution occurred. Hence after separating on a suction funnel it was dissolved in 0.2 n. NaOH and refluxed again on a water-bath for 5 hours. From the cold dark brown solution appeared precipitates on addition of H_2SO_4. The centrifuged supernatant fluid was nearly non-colored. The dark brown centrifugate was dissolved in acetone, neutralized and evaporated. The residual was reprecipitated from dilute alkali by acidification, washed and dried. Analysis revealed that almost no alteration of N- and P-content had happened. N 1.8%, P 4.4%, ash 1.3%.

13. *Isolation of Palmitic Acid.* The evaporated residue of the ethereal solution in 10. was dissolved in 50 cc. of 98% alcohol by heating to 60°C. To the dark brown solution kept boiling a quantity of water, large but hardly sufficient to cause precipitation, was added in portions through the return condenser. The yellow precipitates appearing on cooling in ice were separated on a funnel with suction and washed with 10 cc. of dilute alcohol (3 volumes of alcohol+1 volume of water). The filtrate (dark brown colored) and washing were set aside for a further treatment (s. 14.). The obtained crude palmitic acid amounted to 850 mg. It was taken up again in 30 cc. of boiling alcohol. By addition of 10 cc. water the solution became turbid and a small amount of dark oily deposits adhered to the vessel wall. The solution was decanted out[5] and made transparent by heating with 20 cc. of 95% alcohol and precipitated again by adding water while hot and cooling similar to above. The precipitates were redissolved and reprecipitated more three times to a constant melting point of 61°C. 300 mg. of crystals (needle tufts) were given. The mother liquids of the successive precipitates were distilled in vacuo. When most of alcohol was expelled the fluid changed yellowish and turbid, and a light brown oil (little) floated on the surface. The present turbid fluid was also freed from the oil, diluted with an equal volume of alcohol and heated to 60°C to obtain a clear solution. It was then distilled in vacuo and the precipitates during the distillation were redissolved and reprecipitated as above. Here were given again 300 mg. of crystals having the

same appearance as the above and fusing at 61°C. The both products were mixed together. Nitrogen (Lassaigne), sulfur (Lassaigne) and phosphorus were not detected and in mixed melting point test with palmitic acid no depression occurred. Molecular weight (Rast)$=\frac{1000\times1.01\times40}{10\times15}$ $=269$ (calc., 256). Neutral equivalent:— 20 mg. substance was taken up in 20 cc. of alcohol and titrated with 0.01 n. NaOH (in water). Indicator, phenolphthalein. The blank experiment was carreid out in parallel. N.E.$=\frac{0.020\times1000}{7.51\times0.01}=266$.

14. *Hydrolysis and Following Fractionation of the Substance that had been Separated from Palmitic Acid.* The mother liquid and washing of the crude palmitic acid were distilled at a low pressure. The deep brown oil which remained was 0.8 g. It contained 0.93% nitrogen, and calculated from this nitrogen value minimum molecular weight of the substance presumed as a single compound reached even 1500. Therefore, cleavage of it was made. 730 mg. were heated with 50 cc. of 90% methanol containing 10% (by volume) sulfuric acid for 3 hours (under reflux on a boiling water-bath). To the cooled hydrolysate was added water up to 300 cc., whereby dark oil appeared. The mixture was immediately shaken with changes of petroleum-ether. Tarry matter between the water- and petroleum ether-layer was separated. From the latter layer (brown) remained 0.5 g. of substance (crude unsaturated hydroxyacid) after vacuum-distillation. The former layer (light yellow) was freed quantitatively from H_2SO_4 by means of baryta after addition of 5 cc. of 10% HCl. The solution gave no positive ninhydrin reaction and left no appreciable quantity of substance when distilled in vacuo.

15. *Isolation of an Unsaturated Hydroxy Acid.* The crude product above was taken up[6] in 50 cc. of alcohol. Addition of an equal volume of water gave rise to cloudiness but no precipitation. The cloudiness did not disappear on alkalizing the solution (to pH 9.6 with NaOH). The solution was then shaken with portions of ether, into which nearly all the substance passed over. The extracts were evaporated together and the residual dissolved in 30 cc. of 0.2 n. NaOH in methanol and boiled under reflux for 2 hours (No precipitation occurred). When cold, the solution was diluted with 100 cc. of water and shaken with 50 cc. ether. The ethereal layer was separated, dried with Na_2SO_4 and distilled in vacuo. 0.45 g. brown oily matter remained. This contained no nitrogen (micro Kjeldahl) nor phosphorus and gave no digitonide nor Liebermann-Burchard test for sterol. For quantitative analysis the substance was subjected to distillation at 10.6 mm. Hg. At between 105° and 120°C light yellow oil came over. It left practically no ash on burning, solidified at about 4.5°C and

gave no Fehling reduction test nor Piloty-Stock reaction of ketone. It was insoluble in water, but easily soluble in alcohol, ether, chloroform, benzene and petroleum ether.

Iodine number (Hübl):

28 mg. substance, 1.6 cc. 0.1 n. thiosulfate. Found, 53 $\left(=\frac{1.6\times 0.2\times 100}{20\times 0.028}\right)$.

Hydroxyl number (Normann):

85 mg. substance, 22.4 mg. KOH. Found, 263 $\left(=\frac{22.4}{0.085}\right)$.

Neutral equivalent:

30 mg. substance was dissolved in 20 cc. alcohol and titrated with 0.01 n. NaOH, taking phenolphthalein as an indicator. 3.154 cc. of the alkali were used for the substance. $\text{N.E.}=\frac{0.030\times 1000}{3.154\times 0.01}=951$.

From the values obtained the approximate proportional numbers of OH, double bond and carboxyl are calculated at 4, 2 and 1 respectively.

16. *Examination and Fractionation of the Tarry Substance that Appeared between the Water and Petroleum-ether Layer during the Fractionation in* 14. The tarry substance weighed 0.2 g. and was perfectly soluble in alcohol, chloroform and benzene and did not give the saccharose-conc. H_2SO_4 test for sphingosine. N (micro Kjeldahl), 1.05%. P, 2.2%. Ash, 0.19%. Since it was found not completely soluble in ether, the balance of substance (0.13 g.) was subdivided by means of this solvent. The brownish ether-soluble portion (32 mg.) was free from N and P. The main portion undissolved (dark brown)—**N.P.F. III.**—contained 1.6% N, 3.5% P and 0.28% ash. Acrolein reaction of glycerol was negative. Thus, regarding not only the results of tests but also the quantitative figures of N and P it resembled N.P.F. II (s. 12.), with which, however, it did not look identical because of its somewhat different solubility (soluble in hot water, dilute alkali, alcohol, chloroform, benzene and acetone but insoluble in cold water, dilute acid, ether and petroleum ether). The ether-soluble portion was not further examined, since it was thought from its solubility to be the unsaturated hydroxyacid (15.) that had escaped from elimination.

17. *Supplementary Run: Exclusion of Possibility of Presence of Sphingosine and Glycerol in the Group Lipoid.* 1 g. lots of the group lipoid were taken from the same batch as before and hydrolyzed similarly with H_2SO_4. The appearing oily substance, which solidified when cold (0.9 g.), was collected on a suction funnel (The filtrate was set aside for the search after glycerol) and subjected to hydrolysis again by refluxing on a boiling water-bath with 50 cc. of methanol containing 10% H_2SO_4 for 5 hours. The hydrolysate was cooled and filtered and to the dark brown filtrate (about 50 cc.) was added an equal volume of water, then the mixture was exhausted with petroleum-ether, diluted with 50 cc. of water, evaporated on a boiling

water-bath to about 20 cc. and cooled. No precipitates were given (Sphingosine comes out here, if present). Further condensation of the fluid entailed only charring.

The filtrate above of the lipoid hydrolysate was freed from phosphotungstic acid-precipitable substances as described already (The filtrate from the phosphotungstic acid-precipitates was 60 cc.) and shaken with three 30 cc. portions of an amylalcohol-ether (1:1) mixture and then the saturated $Ba(OH)_2$ added to pH 6.0 with following centrifugation. To the supernatant liquid baryta was supplemented till pH 8.0 of the solution. Centrifuged. After removing excess barium by means of CO_2-gas, the solution was condensed to about 2 cc., and 10 cc. of alcohol were added. Since no precipitation occurred (Barium glycerophosphate is precipitated, if present), the mixture was distilled to dryness. The light yellow remainder also gave no acrolein reaction for glycerol.

Summary.

A preparation of Group A lipoid of human liver was hydrolyzed and fractioned. Separated were glucosamine (as hydrochloride), galactose (as methylphenylhydrazone), choline (as chloroplatinate), palmitic acid, an unsaturated hydroxyacid, a probable keto-alcohol and three substances containing N and P. Moreover qualitative tests suggested the presence of formic acid, tyrosine, xanthine and hypoxanthine or guanine among the cleavage products. Sphingosine, glycerol, glycerophosphoric acid and acetic acid were absent.

A short discussion was made regarding the results.

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H. Masamune.

References and Notes.

1) Matuda, Tohoku J. Exp. Med., this volume, p. 333.

2) Masamune, Tokyo Iji Shinshi (Tokyo Med. Journal), 1949, **66**, 269.

3) Thudicum mentioned the positive Pettenkofer reaction of sphingosine (cited by Thierfelder and Klenk, Die Chemie der Cerebroside u. Phosphatide, Julius Springer Berlin, 1930, p. 47).

4) Cf. Satoh et al., Tohoku J. Exp. Med., 1943, **46**, 189.

5) The oil coherent to the vessel was discarded.

6) The substance dissolved completely.

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Biochemical Studies on Carbohydrates.

CXXXVI. A Hexosamine-containing Carbohydrate and a Glucidamin from Human Liver.

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Tissue of livers from Group A persons, dehydrated and freed from lipids, were treated with water and the extracted matter fractioned by means of glacial acetic acid. From the division that dissolved in this solvent was separated a substance (a glucidamin) involving 12.8% N, 9.2% hexosamine, 10% non-amino sugars as galactose (By the indole, orcinol and α-naphthol reaction developed colors quite coinciding in depth as expressed as galactose) and 7.9% ash. It was non-anti-isohemoagglutinative. In the glacial acetic acid-insoluble division was found an abundant quantity of glycogen. After digestion of it with saliva and dialysis there remained a substance (a carbohydrate) analyzing N 3.5%, hexosamine 10.4%, ash 7.7%. The non-amino sugar content was very high and the three colorimetric methods above gave figures remarkably divergent as expressed as any of glucose, mannose and galactose. The substance inhibited the isoagglutination of A erythrocytes at a dilution of 1:800. $[\alpha]_D = +80$.

Paper partition chromatography suggested that the sugar components of the former product were glucosamine, chondrosamine, galactose, mannose, arabinose and a methylpentose and that the latter contained those sugars[1] and besides glucose, though little, which probably belonged to the remnant of the cleavage products of glycogen. Since tests for pentose and methylpentose resulted in negative on not a large quantity of the substances, the amounts of these two sugars contained are estimated very small. The details of the chromatograms will be published in a separate account.

Experimental.

Human livers from two corpses belonging to Group A (2050 g. in total) were cut into pieces, washed, dehydrated and freed from lipids as already reported.[2] The dry tissue-residue weighing 350 g. was powdered

in an iron mortar and blood vessels and fibers were removed. The balance amounted to 300 g. 250 g. were subjected to water extraction.

1. *Preliminary Extraction.* The material was agitated with 3 *l.* of water for 48 hours and centrifuged. The supernatant liquid was yellow and somewhat turbid and gave strong Molisch reaction. The centrifugate was treated in a like manner more three times. The final supernatant liquid contained Molisch-positive substance scarcely. The extracts were distilled together in vacuo to about 2 *l.* and precipitated by addition of the saturated $Ba(OH)_2$ to a pH of 9.0. The deposit was separated and washed with a dilute baryta solution of pH 9.0. (After washing with alcohol and drying it weighed 8.2 g. This was rejected.) The mother liquid and washing united were acidified to a pH of 4.5 by means of acetic acid and stood one day. A trace of precipitates was centrifuged off and the supernatant liquid (transparent and yellow) dialyzed against running water, until it became nearly neutral, and distilled to a thick solution, and 5 volumes of alcohol added. The precipitates here were dried in a vacuum desiccator (H_2SO_4). The gray powder obtained was 4.7 g. Of it 3.7 g. were agitated with four 50 cc. portions of water (The part undissolved was about 0.1 g.) and on the extracts (3rd and 4th extract were translucent) were the operations with baryta and acetic acid, dialysis, distillation and precipitation with alcohol repeated. 1.8 g. of gray powder were given.

2. *Fractionation by means of Glacial Acetic Acid.* The product was kneaded to a paste with several drops of glycol and then glacial acetic acid (98%) added in small portions until no more precipitation occurred (10 cc. of the precipitant were required). Centrifuged. The supernatant liquid was transparent and brown. The centrifugate was washed with changes of glacial acetic acid, as far as a new washing got colored, and then with ether many times to eliminate acetic acid entirely and dried—Fr. I (Crude Polysaccharide). It was gray powder and the yield was 1.0 g. To the mother liquid and glacial acetic acid-washings above were added 3 volumes of ether. The deposit, too, was washed with ether and dried. The light brown substance obtained—Fr. II (Crude Glucidamin)—amounted to 0.5 g.

3. *Isolation of the Polysaccharide.* The crude polysaccharide was treated thoroughly with 5% trichloracetic acid (One 10 cc. and three 5 cc. portions of this solution were employed) and the insoluble part discarded. To the extracts were added 5 volumes of acetone and the precipitates washed with ether and dried—500 mg.

Since the substance contained much of glycogen (iodine assay), salivary digestion was resorted to for further purification, namely, it was dissolved in 10 cc. of water, adjusted to pH 6.5 with 0.01 n. NaOH and after addition of 0.1 cc. of saliva filtrate, placed in a thermostat at 37°C. Glycogen dis-

appeared in 4 hours. Thereupon 5 volumes of alcohol were added to the mixture. The deposit was dried, washed with portions of glacial acetic acid and then with ether repeatedly (to eliminate acetic acid) and again dried. Next it was taken up in 5 cc. of 0.1 n. HCl. The centrifuged supernatant liquid (The insoluble part was small) was precipitated with 5 volumes of alcohol, with following washing with alcohol and drying. The crop here was about 0.4 g. Iodine test for glycogen negative. However, among the non-amino sugar-components in the product glucose appeared to predominate, because the indole, orcinol and α-naphthol (I) reaction developed colors in the depths most coinciding when expressed as glucose. The colorimetry was made on 0.02% solution of the substance.

Analysis	Reading mm.	Percentage of non-amino sugars expressed as		
		Glucose	Galactose	Mannose
Indole reaction	18.5	55	70	80
Orcinol r.	15.5	65	80	90
α-Naphthol r. (I)	18.0	55	110	138

Thus it was conjectured that no small amount of the cleavage products of glycogen accompanied the polysaccharide had in view. Nitrogen (micro Kjeldahl) 4.0%. Hexosamine 8.7%. Ash 1.04%. To remove the probable contaminant 250 mg. of the substance were shaken with 90% acetic acid for 3 days. And the centrifugate was washed with acetone and then with ether. After drying it was taken up in 5 cc. of 5% trichloracetic acid and the centrifuged supernatant liquid (A little centrifugate discarded) precipitated with 50 cc. of acetone. The deposit was washed and dried as immediately above—96 mg. By the present trial the hexosamine content was raised to 9.2% and the different colorimetric methods gave non-amino sugar values as glucose which were more remote from one another than before:

Analysis	Reading mm.	Percentage of non-amino sugars expressed as		
		Glucose	Galactose	Mannose
Indole reaction	11.5	48	64	67
Orcinol r.	10.5	72	45	48
α-Naphthol r. (I)	12.0	45	91	112

(0.0375% solution of the substance was employed for the determinations)

Nitrogen (micro Kjeldahl) 3.5%. Ash 0.79%. But at this stage the writer became aware that the product was capable of reducing Fehling solution intensively and rotating D-light rightwards strong $\left[[\alpha]_D^{12.5} \text{ (in water)} = \frac{1.44 \times 100}{1.8 \times 1} = +80^\circ\right]$, which were suggestive that the digestion products of

glycogen still remained.

Now the material was become insufficient (40 mg. were the balance) for further processes, the writer was compelled to carry out another run of preparation from the commencement, and from 308 g. of the residue of human A livers after defatting and freeing from fibers, 0.11 g. of substance corresponding to the final product above were obtained (In this case the salivary digestion was continued for 24 hours). N (micro Kjeldahl) 3.7%. Analysis of non-amino sugars was effected on a 0.0425% solution.

Analysis	Reading mm.	Percentage of non-amino sugars expressed as		
		Glucose	Galactose	Mannose
Indole reaction	9.8	52	71	77
Orcinol r.	8.6	82	66	73
α-Naphthol r. (I)	9.5	49	99	118

In order to eliminate the reducing impurities the remainder here (99 mg.) and that in the former run (40 mg.) were dissolved together in 20 cc. of water and dialyzed against changes of water through a collodion membrane. When the solution exhibited no reduction of Fehling solution, it was distilled in vacuo almost to dryness and dehydrated with alcohol and a little anhydrous sodium acetate (as dissolved in alcohol). After washing with alcohol and drying, 65 mg. of substance were recovered. It analyzed: N (micro Kjeldahl) 3.5%, hexosamine 10.4%, ash 7.7%. Non-amino sugars were determined on a 0.0333% solution of it:

Analysis	Reading mm.	Percentage of non-amino sugars expressed as		
		Glucose	Galactose	Mannose
Indole reaction	15.5	41	51	57
Orcinol r.	13.0	57	42	44
α-Naphthol r. (I)	16.0	38	75	96

Chromatography of the substance showed spots corresponding to the expected positions of glucosamine, chondrosamine, galactose, mannose (?), arabinose and in addition glucose (a small spot). Qualitative reactions in a test tube:—It gave no biuret reaction but the strong Molisch. Tests for pentose (with aniline acetate; 2–3 mg. were tested on 1/10 scale), uronic acid (Neuberg-Saneyoshi) and phosphorus (ammonium molybdate test with oxidation) resulted in negative. Hydrolysable sulfur was detected but faintly. Some of these results of test-tube reactions conflict with the findings by chromatography and it is believed that pentose and methyl-pentose are scarce, though not absent. To add furthermore, the product inhibited iso-agglutination of A erythrocytes at a dilution of 1:800.

4. *Isolation of the Glucidamin.* The crude glucidamin (s. 2.) was stirred up in 20 cc. of water and centrifuged. The centrifugate was again

treated with 10 cc. of water. Most of the substance dissolved. The supernatant liquids were united and the saturated $Ba(OH)_2$ added to a pH of 9.0. Some precipitates appeared which were centrifuged off and the supernatant liquid here was acidified with HCl to pH 2.0 whereupon again precipitation occurred. The centrifuged supernatant fluid was furtheron subjected repeatedly to similar alkalization and acidification with separating off the deposits and when no more precipitation took place, the fluid acidified was dialyzed against running water. Followingly it was distilled in vacuo to a small volume and precipitated with alcohol. The deposit (300 mg. when dried) still contained phosphorus so that, after dissolving it in 10 cc. of water, the above operations were carried out over again. The amount of substance decreased to 180 mg. Phosphorus was hardly detected. The final substance was readily soluble in water and markedly precipitable by $(NH_4)_2SO_4$ and sulfosalicylic acid (added to 5%). It gave the positive biuret and Molisch reaction. Analyses: N (micro Kjeldahl) 12.8%, hexosamine 9.2%, ash 7.9%. Of the non-amino sugar values (analyzed on an 0.2% solution) calculated from the results of the color reactions those expressed as galactose agreed most with one another.

Analysis	Reading mm.	Percentage of non-amino sugars expressed as		
		Glucose	Galactose	Mannose
Indole reaction	13.1	8.0	10.5	11.5
Orcinol r.	9.6	15.5	10.0	10.5
α-Naphthol r. (I)	20.0	5.0	10.0	12.5

It possessed no potency inhibitory to the iso-agglutination of erythrocytes. According to the consequence of chromatography the substance contained glucosamine, chondrosamine, galactose, mannose, arabinose and a methylpentose. In a test-tube the presence of methylpentose (Rosenthaler) and pentose (aniline acetate test) was proved only when a big amount of the preparation (10–15 mg. substance in a test on 1/10 scale) was applied. Uronic acid (Neuberg-Saneyoshi) and hydrolysable sulfur ($BaSO_4$ method) were absent.

SUMMARY.

From normal human liver two hexosamine-involving substances, one a carbohydrate and the other a glucidamin, were separated. The carbohydrate was blood-group (ABO system)-active and the glucidamin inert in this respect. Their composition was discussed.

Through the Grant Committee of the Science Council was given a grant from the Education Department in aid to us. H. Masamune.

References and Notes.

1) The presence of mannose in the carbohydrate fraction was uncertain.
2) Matuda, Tôhoku J. Exp. Med., this volume, p. 333.

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On the Cerebro-Spinal Fluid Pressure of Toxemias of Late Pregnancy.

By

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Many investigators (Hofbauer, Begdanow, Dagner, Küstner,[1] Franco,[2] Garrone, Schockart, Joseph,[3] Kuji,[4] Nakajima,[5] Toyoshima,[6] Terakawa,[7] Kobayashi-Onagi,[8] Uchino,[9] Shinohara et al) reported that fits of eclampsia could be brought about clinically by injection of posterior pituitary hormone (P.P.H.) to the women suffering from toxemia of pregnancy, Ohno[10] reported that 11 out of 2164 patients of eclampsia admitted into principal hospitals in Japan were caused by P.P.H.

We have also experienced two similar causes. The mechanism, however, is yet unknown.

It is generally accepted that the spinal fluid pressure increases in the severe toxemia of pregnancy, especially in the eclampsia, and eclamptic convulsions can be controlled by pressure depressing of spinal punctures. On the other hand, P.P.H. in the blood of toxemias, according to Anselmino-Hoffman,[11] increased in amount. Therefore I have tried to study the influence of this hormone upon the fluid pressure of toxmia and the relation between the two. Furthermore, if possible, to clarify the mechanism of eclamptic fits caused by P.P.H.

P.P.H. Influences upon the Spinal Fluid Pressure.

Weed and Cushing,[12] Becht and Matill,[13] Konishi,[14] Tajitsu,[15] Watanabe[16] et al, through animal experiments of the intravenous injections, found the elevation of fluid pressure, Dixon and Halliburton[17] found the decreasing or no change, and Endo recognized the slight elevation or no change. These results, therefore, are unstable. There is no report of elinical investigation about this subject.

An apparatus used in this experiment is the Yamada-Sata's.

Avoiding carefully the influences from other causes (i.e. abdominal pressure, muscular rigidity, emotional hyperirritability and others), and by

reading the almost stable fluid pressures of the tested women lying on the right side for more than 5 minutes after spinal puncture, I injected into them 0.1 to 0.15 of Atonin—1 to 1.5 I.U.—(P.P.H.) intravenously to read the changes in fluid pressures. Stimulus of injection was made as slight as possible, pressure reading was done every 5 seconds during the first 5 minutes and thereafter every 10 seconds. The curves in Fig. 1. (Table I) were plotted from the results of two healthy non-pregnants and of 16 normal pregnants.

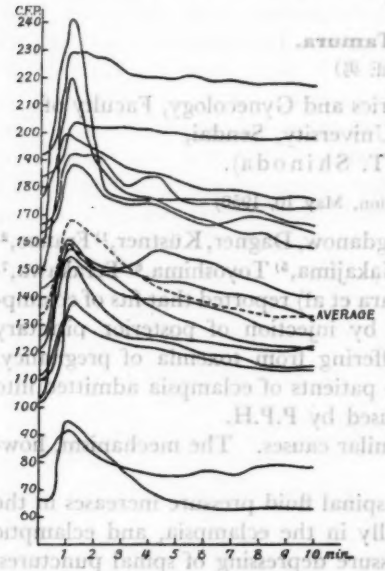

Fig. 1. Influences of P.P.H. upon the spinal fluid pressure. (Changes of the pressure after injections of atonin.)

Intravenous Atonin injections always increased rapidly the fluid pressures in every case, and the pressures arrived at the highest about 50 to 80 seconds (average 62 seconds) after the injections. Extensions of the increases were 10 to 67 mm. (average 34.2 mm.). Afterwards, somewhat rapid at first and gradual decreases secondly took place, then it returned to the beginning at last after 2½ to 16½ min. (average 9 min. 54 sec.).

The time to arrive at the highest had nothing to do with either presence of pregnancy or the months of pregnancy. It seems that the time to recover is generally longer in the last half term of pregnancy than in the first half.

The elevation degree has something to do with an amount of Atonin, but not always with the initial fluid pressure and the time to recover.

The Fluid Pressure of Toxemia of Pregnancy, Especially of Eclampsia.

As to the fluid pressure of toxemias of late pregnancy, several authors (Kylin and Brenning,[18] Perz, Eskuchen and Lickint,[19] Lockemann,[20] Heynemann, Wieloch,[21] Kubota, Saito,[22] Mizuhara,[23] Kurita, Sasaka,[24] Takata et al) stated that it has increased, but about the pressure of healthy pregnants there is no settled view among these authors. As a normal range, Osato-Hioki decided it to be between 100 and 125 mm., but other authors (Quinke, Kure, Sata, Kurita et al) decided as a normal limit to be 150 mm. or 200 mm. to Becker, some healthy one had a fluid pressure

TABLE I.

No.	Name	Age	Diagn.	Pressure of cerebrospinal fluid: Initial mmH$_2$O	Elevation mmH$_2$O	Elevation percentage	Max.	Time till max. pres. (h.)	Duration of elevation
1	K.H.	20	Cervixcatarrh	122	38	31.1	160	60″	8′40″
2	I.S.	27	2 P.M.	122	22	19.6	134	55″	11′10″
3	J.U.	25	2 „	126	34	26.9	160	60″	9′10″
4	T.S.	27	3 „	82	10	12.1	92	60″	2′45″
5	K.T.	32	3 „	164	25	15.2	189	1′10″	10′50″
6	H.A.	31	6 „	103	52	50.4	155	60″	15′20″
7	H.S.	21	6 „	160	40	25.0	200	50″	6′40″
8	H.O.	18	6 „	167	25	14.9	192	60″	9′50″
9	T.T.	21	6 „	127	23	18.1	150	1′20″	9′10″
10	M.K.	22	7 „	192	39	20.3	231	50″	16′10″
11	S.I.	20	8 „	103	44	42.7	152	1′15″	15′20″
12	T.I.	20	9 „	125	37	29.6	162	55″	13′10″
13	T.S.	33	10 „	143	15	10.4	158	60″	9′00″
14	S.M.	25	Hypoplasisuteri	177	65	36.7	242	60″	2′20″
15	S.K.	38	3 „	165	67	40.6	232	60″	8′10″
16	S.K.	21	5 „	66	30	45.4	96	1′05″	4′40″
17	U.A.	22	7 „	110	29	26.3	139	1′05″	10′40″
18	T.O.	37	10 „	184	20	10.8	204	1′05″	14′50″
			Average	134.8	34.2	26.4	169.3	1′02″	9′54″

Remarks: In nos. 14 to 18 0.15 cc. of atonin (1.5 I.U.) were injected, in the other cases 0.1 cc. of atonin.

of 200 mm., as shown in Table I. Also, individual differences of the pressures are markedly great. Therefore to conclude that toxemias have increased fluid pressures, further investigations are required.

From my results founded on the investigations of 33 normal pregnants from the 2nd to the 10th pregnant months, as shown in Table II, an average pressure is 133.39 mm. Among them there are 10 cases of over 150 mm. and 11 cases of below 115 mm.; the highest is 192 mm., the lowest is 66 mm., and also very marked are individual variations.

Our criteria for toxemias is as follows: 1. Albuminuria of over 0.5% (Esbach)—Type A. 2. Edema beyond the lower abdominal region—Type O. 3. Hypertension over 150 mmHg.—Type H. 4. Two or three of these symptoms—Type AO, OH, AH and AOH.

The pressures under each type of toxemias are shown in Table III.

TABLE II.

The Cerebrospinal Fluid Pressure of Normal Pregnants.

No.	Name	Pregnancy (month)	Crebrospinal fluid pressure
1	K.H.	II	122 mmH_2O
2	J.U.	II	126
3	K.M.	II	165
4	T.S.	II	113
5	T.H.	III	114
6	M.O.	III	162
7	S.H.	III	145
8	M.Y.	III	97
9	T.S.	III	82
10	S.M.	III	165
11	K.T.	III	164
12	H.S.	III	142
13	H.T.	IV	127
14	I.S.	IV	187
15	Y.K.	V	102
16	K.S.	V	172
17	S.K.	V	66
18	H.O.	VI	167
19	S.S.	VI	160
20	T.A.	VI	103
21	M.M.	VI	105
22	T.I.	VI	168
23	T.Y.	VI	110
24	H.T.	VII	79
25	U.T.	VII	145
26	M.S.	VII	121
27	S.H.	VIII	126
28	T.M.	VIII	124
29	K.T.	VIII	192
30	K.S.	IX	179
31	K.I.	IX	104
32	S.I.	IX	125
33	T.I.	X	143
		Average	133.39

The highest is 330 mm., the lowest is 151 mm. (average 216.43 mm.) and there is no case of less than 150 mm. Because the pressures over 192 mm. (the highest figure in normal pregnants as above described) amounted to 15 out of 23 cases (65.2%). The fluid pressures of toxemias are evidently higher than of normal pregnants. As shown in Fig. 2, the Type AOH and the Type AH have many cases of increased fluid pressures. Especially in eclampsias, the highest is 290 mm., the lowest is 181, mm. mostly the pressures of over 200 mm. Evidently, therefore, the severer becomes a toxemia, the higher increased its fluid pressure; Much more in eclampsia.

Relation Between the Fluid Pressure and the Convulsion.

Although, from the observations above described, it was recognized that toxemic, especially eclamptic patients showed abnormal elevations of the pressures and P.P.H. increased their fluid pressures. To know if a rapid elevation of the pressure really causes convulsion or not, by animal experiments (rabbit) I investigated a relation between a rise of the pressure and the cardiazol convulsion threshold.

Beforehand I determined the cardiazol convulsion threshold—the minimum dosage to cause convulsion—of rabbit (body weight of about 3 kgm.), and then after introducing 2 cc. of Ringer's saline solution into the ventricles by an occipital puncture. I injected into the rabbit intravenously cardiazol solution of 0.02 to 0.05 cc. less than the threshold to examine an occurrence of convulsion fits. It is needless to say that this dosage only of cardiazol does not cause the convulsion.

Rabbit No. 1. ♂, wt. 3,000 gm.

By an intravenous injection of 0.35 cc. of cardiazol no reaction was seen, by injection of 0.4 cc. slight irritability, and by injection of 0.45 cc. of fits of convulsion occurred. That

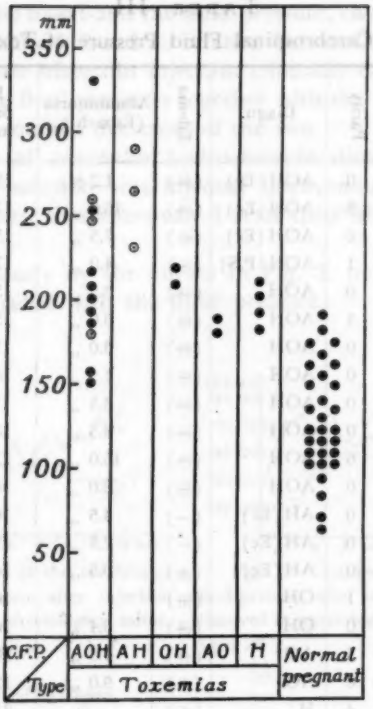

Fig. 2. The spinal fluid pressure of toxemias, compared with those of healthy pregnants. ⊙ means eclampsia.

is, the threshold is 0.45 cc.

After an occipital injection of 2 cc. of saline solution, 0.4 cc. of cardioxzol(i.v.) caused the rabbit marked fits of convulsion.

Rabbit No. 2. ♂, wt. 3,100 gm.

The threshold is 0.27 cc., and by 0.25 cc. of cardiazol (i.v.) after an occipital injection the fits occurred.

Rabbit No. 3. ♀, wt. 2,950 gm.

The threshold is 0.3 cc., and 0.27 cc. of cardiazol (i.v.) produced the marked fits.

From these results, I have evidence that the increased spinal fluid pressures are apt to cause the convulsion fits.

The Mechanism of a Rise of the Fluid Pressure Caused by an Intravenous Administration of P.P.H.

About the mechanism of a rise of the fluid pressure, causation by hypertension is most extensively discussed. Although Pjaundler, Berg-

TABLE III.

The Cerebrospinal Fluid Pressure of Toxemias.

No.	Name	Age	Parity	Diagn.	Edema	Albuminuria (Esbach ‰)	Blood pressure mm Hg	Cerebrospinal fluid pressure mm H_2O
1	T.K.	25	0	AOH (Ec)	(卌)	1.2 ‰	150-120	181
2	M.A.	41	5	AOH (Ec)	(卌)	16.0 „	170- 84	254
3	N.O.	22	0	AOH (Ec)	(卌)	7.5 „	155-130	250
4	M.I.	28	1	AOH (P.S)	(卌)	4.0 „	178-110	251
5	Y.O.	27	0	AOH	(++)	3.5 „	158- 90	220
6	M.O.	27	1	AOH	(卌)	8.0 „	152- 96	192
7	M.S.	25	0	AOH	(卌)	3.0 „	156-100	200
8	K.G.	21	0	AOH	(卌)	1.5 „	182-108	151
9	K.Y.	24	0	AOH	(卌)	3.5 „	158-100	211
10	H.I.	27	0	AOH	(卌)	8.5 „	180- 90	152
11	T.H.	36	6	AOH	(卌)	15.0 „	228-178	330
12	S.M.	34	0	AOH	(卌)	8.0 „	180-130	184
13	H.S.	22	0	AH (Ec)	(−)	4.5 „	165- 80	262
14	F.S.	24	0	AH (Ec)	(−)	2.5 „	192-125	290
15	M.H.	22	0	AH (Ec)	(±)	0.5 „	142- 92	235
16	N.I.	31	1	OH	(卌)		160- 80	221
17	M.K.	24	0	OH	(卌)	0.4 „	163-120	216
18	T.O.	37	5	AO	(++)	4.3 „	104- 70	184
19	M.I.	27	0	AO	(卌)	9.0 „	120- 90	187
20	N.G.	33	4	H	(±)		215-165	217
22	I.H.	22	0	H	(−)		156- 80	207
23	K.A.	24	0	H	(−)	0.15 „	190-100	185
							Average	216.43

Remarks: Ec=eclampsia, P.s.=premature separation of placenta.

mann, Ziegler, Hill, Edler, Nagashima, Suenaga[25] et al found a close correlation between the fluid-and the blood-pressures, Dixon and Halli burton,[17] Miwa, Kawaguchi, Harada et al reported that the blood pressure is only one of the factors influencing the fluid pressure, which is under control of a volume of the brain vessels.

Correlation between the fluid pressure and the blood pressure was examined as follows:

1) *Adrenalin.*

As to relation between the fluid pressure and an adrenalin injection numerous papers are found. Dixon and Halliburton[17], Becht, Matill[13], Konishi,[14] Tajitsu,[15] Endo and others recognized by animal experiments

a transitory rise of the blood-and the fluid-pressure, caused by an adrenalin injection, but Watanabe[16] fouud no relation between them. Tamura and Oho[26] stated that an adrenalin injection clinically caused in most cases transitory rises of the fluid pressures together with the blood pressures, but occasionally caused marked decreases of the two.

After 0.02 mg. of adrenalin hydrochloride dissolved into 5 cc. of physiological saline solution was injected intravenously into each of 5 healthy non-pregnants, simultaneously I read their blood- and fluid-pressures.

As shown obviously by the curves in Fig. 3. not always the blood pressure rises in parallel with the fluid pressure.

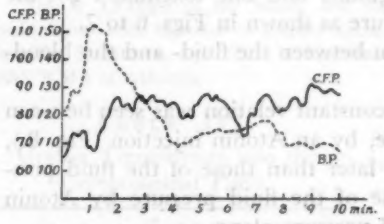

Fig. 3. The changes of the cerebrospinal fluid and blood pressure after injection of adrenalin as dissoved in physiological saline. Case No. 5. Kisara aged 22 y.

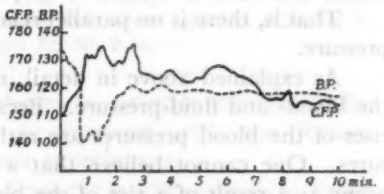

Fig. 4. Changes of the blood- and the fluid-pressure after injection of pilocarpine as dissolved in physiological saline. Case No. 1. Aged 28 y.

2) *Pilocarpine and Atropin.*

The papers about effects of pilocarpine and of atropine on the cerebrospinal fluid pressure are relatively rare, one can find only animal experiments by these agents (Dixon and Halliburton,[17] Watanabe,[16] Tajitsu,[15] Endo, Fujii, Kawaguchi, Kataoka et al.). Their effects and effect mechanisms also are yet unsettled.

I injected, therefore, 1.2 to 0.8 mg. of pilocarpine hydrochloride and 0.1 mg. of atropine sulfate, both dissolved into 5 cc. of saline solution, into each of 7 healthy non- pregnants.

As it's obvious in Fig. 4. to Fig. 5. changes of the blood- and the fluid-pressure all run contrary to each other.

3) *Sodium nitrite* ($NaNO_2$).

Because blood vesseles, especially of the brain are very sensitive to sodium nitrite and amyl nitrite, there is a lot of reports about effects of these agents upon the blood pressure, but few about that upon the fluid pressure, and its experimental results also disagree with each other. Amyl nitrite, Biedl and Reiner reported, causes a fall of the blood pressure and a rapid rise of the fluid pressure, without relation to the blood pressure.

Dixon and Halliburton[17] investigated that by this agent the blood pressure decreased and the fluid pressure also decreased at first but afterwards quickly increased, thenceforth both of them finally returned to the beginning almost simultaneously. Bencht, Koishi,[14] and Tajitsu[16] also ascertained a rise of the fluid pressure together. On the other hand, Tamura and Oho[26] examined the effects of sodium nitrite and of amyl nitrite clinically in addition to animal experiments, and concluded that, although a marked rise of the fluid pressure occurred on an animal, not always in a same manner as on a human being. No evident change was seen in the latter.

By an intravenous injection of 20 cc. of 2 per cent sodium nitrite solution to each of two healthy non-pregnants and one toxemia, I got the curves of the blood- and the fluid-pressure as shown in Figs. 6 to 7.

That is, there is no parallel relation between the fluid- and the blood-pressure.

As explained above in detail, no constant relation was seen between the blood- and fluid-pressure. Because, by an Atonin injection (Fig. 8.), rises of the blood pressures are rather later than those of the fluid pressures. One cannot believe that a rise of the fluid pressure by Atonin occur as a result of a rise of the blood pressure alone.

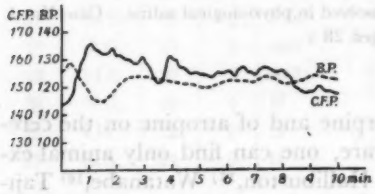

Fig. 5. Changes of the blood- and the fluid-pressure after injection of atropine as dissolved in physiological saline. Case No. 7. Aged 27 y.

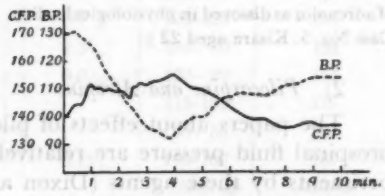

Fig. 6. Changes of the blood- and the fluid-pressure after injection of sodium nitrite. Case No. 2. Kobayashi aged 31 y, non-pregnant.

Then to study the mechanism of a hypersecretion or of a hyper-permeability of the cerebrospinal meninges, 11 non-pregnant and 3 pregnant women (all negative to Wa-R) were injected 20 cc. of 10 to 20 per cent solution of uranin (Takeda Pure Chemical Ltd.) intravenously in about one minute. 20 minutes after the injection, by lumbar puncture about 5 cc. of the cerebrospinal fluid was withdrawn to estimate its uranin quantity colorimetically by comparison with the standard solution. 7 to 10 days after that, when its complications (headache, meningism etc.) vanished entirely, the spinal fluid was withdrawn 20 minutes after an intravenous injection of 0.6 cc. of Atonin (6 I.U.) with uranin solution similarly

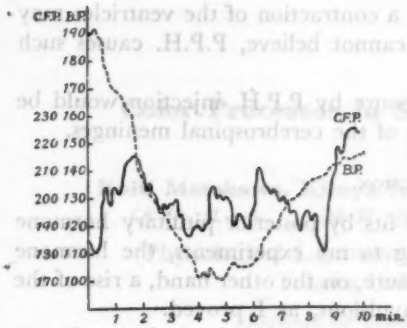

Fig. 7. Changes of the blood- and the fluid-pressure after injection of sodium nitrite. Case No. 3. Aizawa, aged 24 y, a toximexia (10th P.M.) of AH.-type.

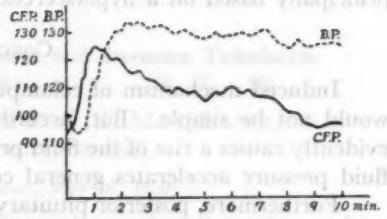

Fig. 8. Changes of blood- and fluid-pressure after atonin injection. Case No. 5. Aged 32, 5th P.M.

TABLE IV.

Uranin Transference into the Cerebrospinal Fluid.

No.	Name	Uranin concentration of spinal fluid	
		Withont atonin	With atonin
1	T.S.	62×10^{-5}	45×10^{-5}
2	S.T.	63× „	60× „
3	E.N.	70× „	62× „
4	S.T.	62× „	58× „
5	Y.O.	60× „	58× „
6	O.K.	62× „	58× „
7	M.Y.	72× „	63× „
8	S.W.	62× „	52× „
9	Y.D.	63× „	58× „
10	M.U.	62× „	59× „
11	T.K.	68× „	59× „
12	O.T.	58× „	55× „
13	K.U.	28× „	25× „
14	K.O.	21× „	19× „

Remarks...1) In the cases nos. 13 and 14, 20 cc of 20 per cent solution and, in the remaining, 20 cc of 10 per cent solution. were injected 2) Nos. 10, 11, and 12 were pregnants respectively in the both, 8th, and 3rd p.m.

as above, to estimate colorimetically and to compare with the former control experiments.

From the results as shown in Table IV, I knew that in every case the concentration of uranin was markedly elevated by Atonin. That is, Atonin uranin solution can be transfered into the cerebrospinal cavity in a larger quantity. But about whether the fact is based on a hypersecretion of the cerebrospinal meninges or on a hyperpermeability caused by its physico-chemical changes, one can not jump to a hasty conclusion.

If in case of physico-chemical changes, the rate of uranin increasing in two cases by 20 per cent solution should be greater than in the other by 10 per cent solution. Table IV. shows the contrary results to this assumption. It might be, therefore, concluded that a hypersecretion of the cerebrospinal meninges, principally of the chorioid plexus played the chief role.

On the other hand, even though a contraction of the ventricles may also increase the fluid pressure, one cannot believe, P.P.H. causes such a contraction.

Therefore, a rise of the fluid pressure by P.P.H. injection would be principally based on a hypersecretion of the cerebrospinal meninges.

Conclusion.

Induced mechanism of eclamptic fits by posterior pituitary hormone would not be simple. But, according to my experiments, the hormone evidently causes a rise of the fluid pressure, on the other hand, a rise of the fluid pressure accelerates general convulsions, as I proved.

Furthermore, posterior pituitary hormone in the blood of toxemias, as generally noticed, increases in quantity. I have ascertained myself a marked rise of the fluid pressure of toxemia, too.

From these standpoints, I would stress a provocation of eclamptic fits by the hormone based on a rapid rise of the cerebrospinal fluid pressure by an injection of this agent, not resulted from a rise of the blood pressure but principally from a hypersecretion of the cerebrospinal meninges.

References.

1) Küstner, Deut. med. Wschr., 1923, **58**, 8.
2) Franco, Ber. ü. Geb. & Gyne., 1938, **37**, 690.
3) Joseph. Ber. ü. Geb. Gyne., 1936, **32**, 428.
4) Kuji, Sanka Fujinnka, 1936, **4**, 466.
5) Nakajima, Sanka Fujinka, 1938, 345.
6) Toyoshima, Jikken Iho, 1936, **269**, 489.
7) Terakawa, Sanka Fujinka, 1940, **8**, 364.
8) Kobayashi-Onagi, Nihon Fujinka Gk. Z., 1940, **35**, 1194.
9) Uchino, Sanka Fujinka, 1940, **8**, 768.
10) Ohno, Rinsho Sanka, 1939, **14**, 98.
11) Anselmino-Hoffmann, Arch. f. Gyne., 1931, **147**, 621.
12) Weed and Cushing, Am. J. Physiol., 1915, **36**, 77.
13) Becht and Mantill, Am. J. Physiol., 1920, **51**, 126.
14) Konishi, Osaka Igk. Z., 1928, **27**, 2919.
15) Tajitsu, Tokyo Igk, Z., 1929, **43**, 507.
16) Watanabe, Shinkei Z., 1929, **30**, 502.
17) Dixon and Halliburton, J. Physiol., 1913, **47**, 215.
18) Kylin and Brenning, Z. exper. Md., 1933, **90**, 328.
19) Eskuchen and Lickint, Münch. med. Wschr., 1929, **76**, 480.
20) Lockemann, Münch. med. Wschr., 1906, **53**, 299.
21) Wieloch, Arch. f. Gyne., 1937, **132**, 296.
22) Saito, Nihon Fujinka GK. Z., 1930, **25**, 189.
23) Mizuhara, Kinki Fujinka Z., 1928, 11, 1079.
24) Sasaka, Tokyo Iji Ss., 1938, 1657.
25) Suenaga, Kumamot Igk. Z., 1936, **12**, 847; 1937, **13**, 883.
26) Tamura and Oho, Nihon Naika Gk. Z., 1930, 18, 391.

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Color Processes in Single Retinal Elements

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In our preceding papers[1)2)3)4)] it was shown that the same mode of analysis as used on the human eye by Motokawa proved to be still useful on excised frog's eyes when action potentials of the optic nerve instead of electrical phosphenes were taken as the index. By this method three kinds of color processes were distinguished in the frog's retina. Each process has its own time constant and can be characterized by it. However, the question arises as to whether the color processes distinguished in this way may be considered as elementary retinal processes or represent merely some group averages. This question will be attacked most adequately by means of the micro-electrode technique recently developed by Granit[5)6)] and others.

We went, therefore, to the single-spike technique not only to answer the question raised above, but also to be able to make the data obtained by Motokawa's method of analysis directly comparable with those obtained by Granit and his collaborators.

Experimental.

Method and Procedure.

As experimental materials excised eyes of Japanese toads were used. After removal of lens and cornea the eye was placed on a pad of Ringer-soaked cotton lying on a large plate of silver which served as a leading-off electrode (see Fig. 1). Micro-electrodes used in the present investigation consisted of a 40 micra silver wire, round which a glass capillary was fused in such a manner that the tip formed a well round point. The electrode was placed by means of a screw of a microscope-stage gently onto the retina. Discharges of retinal elements were detected by means of a four stage R-C coupled amplifier connected with a cathode ray oscillograph and a loudspeaker. The impulses were simultaneously listened to and observed on the screen of the oscillograph. When the tip of the micro-electrode came sufficiently close to a retinal elements, what was known by

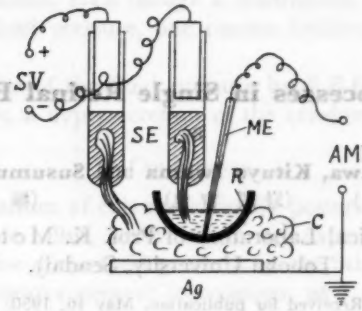

Fig. 1. Experimental arrangements. Ag: silver plate. AMP: amplifier. C: Ringer-soaked cotton. ME: micro-electrode. R: retina. SE: stimulating electrodes of Zn-$ZnSO_4$-gelatine type. SV: stimulating voltage.

a sudden increase in the base-line noise of the loudspeaker, the whole retina was illuminated to see whether or not well-isolated spikes appeared in response to the illumination.

Stimulating electrodes used were non-polarizable electrodes of Zn-$ZnSO_4$-gelatine type. For stimulation a constant current was made to pass through the retina from outside to inside for two seconds, and impulses at the make of the current were chosen as the index. When the electrical sensitivity of the element was found too low, that element was abandoned to seek for another more excitable element.

The procedure of experimentation was as follows: The electrical threshold at the resting level was determined after a preliminary dark adaptation of about 15 minutes. The retina was thereafter exposed to white light for two seconds, and then stimulated electrically every 30 seconds till about 10 minutes after removal of the pre-illuminating light. In this series of experiments it was tried to adjust the intensity of each electrical stimulus to just the threshold value at each moment in order to obtain a just perceptible response. In determination of thresholds two persons were engaged, of whom one was watching the screen of the oscillograph and the other listening in the loudspeaker. They were ordered to inform the experimenter who was adjusting the intensity of stimulating currents, of each response with the following three kinds of expression: no response, just perceptible and above threshold. These were denoted by signs −, + and ++ respectively in the record. If the reports from the two persons did not agree with each other it was marked by two kinds of signs. When successful trials in which each applied voltage was found to be of just threshold strength at the moment, were numerous enough, we constructed an excitability curve from the data obtained in such a single series of measurements. If trials were not successful enough to allow the

construction of an excitability curve, the same procedure—pre-illumination and then electrical stimulation at intervals of 30 seconds—had to be repeated once more. Repetition of the same procedure proved useful in experiments on preparations with the whole optic nerves, for physiological conditions of most preparations remained fairly constant for a long time, but in experiments on single elements or a restricted number of elements conditions were not always favorable enough to allow repetition of the procedure. Therefore we endeavored to finish measurements in a single or at most two series of experiments. For a trained experimenter it was not so difficult, as it might appear, to adjust the intensity of each stimulus to the threshold strength.

Results.

Fig. 2(a) shows well-isolated impulses from a single retinal element in response to illumination with white light, and three single spikes elicited by an electric stimulus are shown in Fig. 2(b). The noise-less part of this record is due to shock artefacts.

We could in this experiment confirm the fact found by Hartline[7] and Granit[8] that retinal elements fall into the three main types, i.e. on-element, off-element and on-off-element. Since the number of our observations on pure on- and off-elements is not sufficient, only the data obtained from on-off-elements will be reported in the following. In Fig. 3 some typical excitability curves are illustrated. As can be seen in the figure, the curves obtained from single elements or a restricted number of elements show much simpler time courses than those obtained from preparations with the whole optic nerves. The curves for white light obtained from such preparations with the whole optic nerves show usually three humps R, G and B at about 1.5, 3 and 5 minutes at room temperature. And we see that the curves at single elements have crest times just corresponding to any one of the crest times of the three elevations R, G and B. As illustrated in the lowermost curve of Fig. 3, some elements, however, showed distinct two elevations, although their responses to stimuli consisted of at least apparently well-isolated spikes. In such cases, the crest time of one elevation was always found about the same as that of the crest time of the B elevation, while the crest time of the other elevation

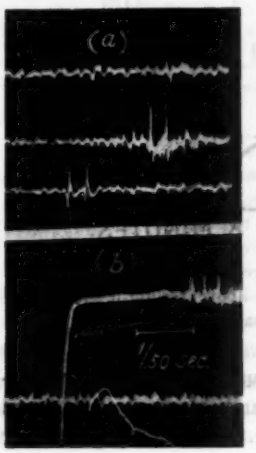

Fig. 2. Spikes from single retinal elements in response to an optic (a) and to an electric stimulus (b). Uppermost and lowermost records controls showing noise-level.

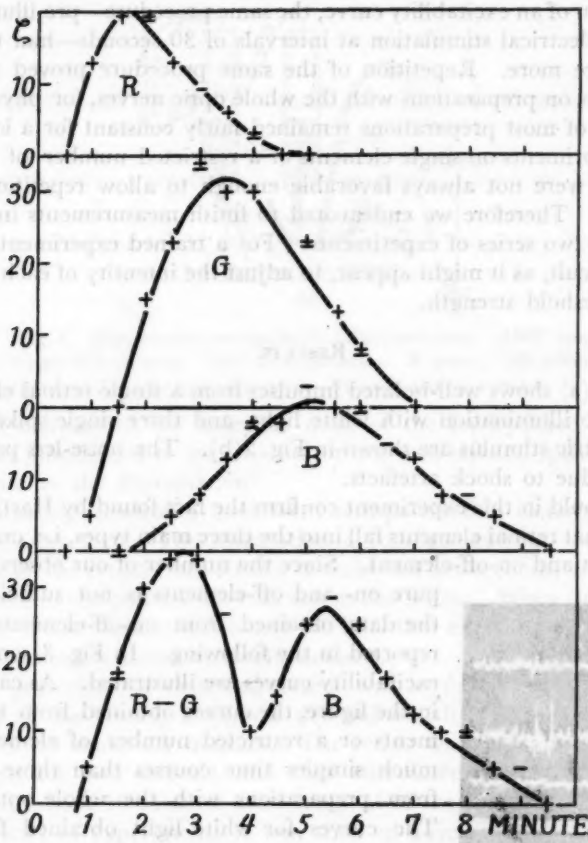

Fig. 3. Excitability curves for white light of some retinal elements. Ordinate: percentage increases in electrical excitability above resting level. Abscissa: time in minutes after cessation of pre-illumination.

corresponded to that of the R or G, or took a value intermediate between R and G. We have not yet encountered such elements as showed separate two elevations corresponding to R and G. This may partly be due to the circumstances that the error of measurements is too great to distinguish the two elevations from each other which are located temporally so little apart, but there is some evidence to show a strong fusion tendency of the two components R and G; for example, the two components frequently appear fused together in the excitability curve for white light obtained from a preparation with the whole optic nerve, where accuracy

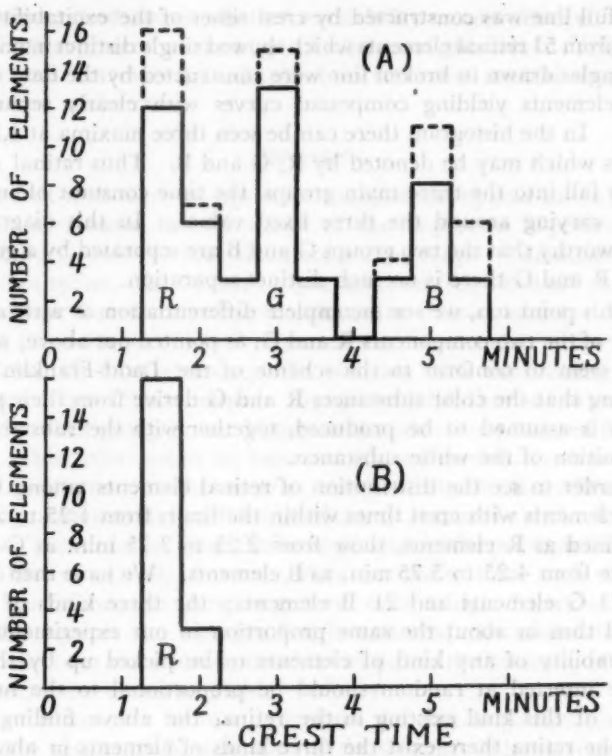

Fig. 4. Histograms concerning crest times of excitability curves of single retinal elements. Rectangles drawn in broken lines were constructed by crest times of elevations of compound excitability curves with two distinct maxima. (A): white pre-illuminating light. (B): red pre-illuminating light.

of measurement is much higher than in experiments on single elements.

The compound nature of the curve manifested itself in some elements also in such a fashion that the curve took a long lasting time course, though it had a single maximum. For example, a curve showing a distinct maximum at 3 minutes took a considerably protracted course in its descending branch, as if a weak B component were participating in that part of the curve. Such an element probably consisted of two components G and B of which the G component was apparently predominant. In the following statistical treatment, however, we treated such an elements as belonging to the G group without taking the accompanying B component into account, because the criterion of the treatment was the crest time.

The statistical data are shown graphically in Fig. 4(A). The histo-

gram in full line was constructed by crest times of the excitability curves obtained from 51 retinal elements which showed single distinct maxima, and the rectangles drawn in broken line were constructed by the data obtained from 7 elements yielding compound curves with clearly separate two maxima. In the histogram there can be seen three maxima at 1.5, 3 and 5 minutes which may be denoted by R, G and B. Thus retinal elements obviously fall into the three main groups, the time constant of individual elements varying around the three fixed values. In this diagram it is also noteworthy that the two groups G and B are separated by a gap, while between R and G there is no such distinct separation.

In this point too, we see incomplete differentiation or a strong fusion tendency of the two components R and G, as pointed out above, and these findings seem to conform to the scheme of the Ladd-Franklin's theory advocating that the color substances R and G derive from their precursor Y which is assumed to be produced, together with the substance B, by decomposition of the white substance.

In order to see the distribution of retinal elements among the three groups, elements with crest times within the limits from 1.25 to 2.25 min. were defined as R elements, those from 2.25 to 3.75 min. as G elements and those from 4.25 to 5.75 min. as B elements. We have then 23 R elements, 21 G elements and 21 B elements; the three kinds of elements appeared thus in about the same proportion in our experiments. Since the probability of any kind of elements to be picked up by the microelectrode inserted at random should be proportional to the number of elements of this kind existing in the retina, the above finding suggests that in the retina there exist the three kinds of elements in about equal number. We used white light as pre-illuminating light in the experiment stated above. If any colored light is used for pre-illumination the three kinds of elements will never appear in the same proportion. This is shown in the following experiment, in which we illuminated the whole retina with red light from a color filter transmitting a range of wave-lengths from 620 to 740 mμ, and determined excitability curves as before on 19 isolated single elements. The data are shown as a histogram in Fig. 4(B). As can be seen in this figure, 16 elements out of 19 have crest times ranging from 1.25 to 1.75 minutes, and the crest times of the other three elements fall into the range between 1.75 and 2.25 min., thus all the elements isolated under this condition belong to the R group.

It seems to be almost unnecessary to carry out similar troublesome experiments with other colored lights, because the relation between crest times and wave-lengths of lights used for pre-illumination has already been well-established in the preceding experiment on excised frog's eyes as well as in the human experiments. These experiments are sufficient to answer

the question raised above whether excitability curves obtained in human experiments represent an average of elemental processes. Yes, they are averages and that averages obtained in the most natural way without resort to any arithmetics.

Discussion.

The fact that three groups of retinal elements appear in the histogram of crest times, seems to correspond to the important experimental fact provided by Granit[9)10)] that there are three groups of modulators in the mammalian retina. In Granit's experiment on frog's retina[6)] the "blue" elements were found exceedingly difficult to isolate, whereas in our experiment the isolation of the B elements was found much easier than that of the other kinds of elements. In contrast with the incompletely differentiated "blue" maximum in the spectral distribution curve reported by Granit, the B group in our histogram stands clearly isolated from other groups. This difference in the behavior of the B modulators is presumably due to the difference in the mode of analysis. By the Granit's method of measuring photosensitivity of retinal elements it must be difficult to isolate the "blue" modulators from the most photosensitive scotopic dominators. Our method of analysis, however, does not suffer from such difficulties, because the modulators show in general such a high degree of electrical excitability against the scotopic dominators that the modulators always predominate over the scotopic dominators,unless the intensity of illumination is below the cone-threshold.

There is another minute but none the less important discrepancy between the results by Motokawa and by Granit: The R spectral locus of the human eye determined by Motokawa[1)] shows a second hump in the violet end of the spectrum, while there is no such second rise in the R locus of the cat's eye determined by Granit.[9)10)] The question now arises as to whether this discrepancy is due to the difference of species or to the difference of the methods. In this respect, Donner's work[11)12)] on the cat's retina is much worthy of attention. This author has revealed that the discharge frequency of retinal elements as a function of time is different according to the wave-lengths, but depends little on intensities of light. His three curves obtained by plotting the frequency maxima for red,green and blue agree with the spectral loci obtained by Motokawa from the human retina even to the extent of reproducing the hump in the violet part of the spectrum. Donner and we are, so to speak, looking on both sides of one and the same shield, measuring spike-frequencies on one hand and electrical excitability on the other hand. In view of the situation that the second rise of our R locus in the violet end well conforms to the results of color mixing experiments performed by a number of investigators, there

is no doubt that our methods utilizing time constants of retinal elements for analysis provide the most reliable results.

Summary.

Color processes at single retinal elements or at a restricted number of elements of Japanese toads were investigated with the aid of microelectrodes, taking well-isolated spikes as the index. The electrical excitability of a single retinal element as a function of time after cessation of pre-illuminating white light shows a maximum at 1.5, 3 or 5 minutes. These values of crest time correspond respectively to those of the R, G and B elevations which are found on the excitability curve for white light determined with action potentials of the whole optic nerve as the index. From retinal elements responding with isolated spikes to optic and electric stimuli sometimes an excitability curve having two distinct humps was obtained.

A histogram concerning crest times of excitability curves of these isolated elements shows that there are three groups of retinal elements. The number of elements belonging to each group was 23 for the R group, 21 for the G group and 21 for the B group. When red light was used instead of white light for pre-illumination all the 19 elements tested in this series of experiments showed excitability curves of the R type. The retinal elements so far studied are to be identified with Granit's modulators.

References.

1) Motokawa, J. Neurophysiol., 1949, **12**, 291.
2) Motokawa and Iwama, Tohoku J. Exp. Med., 1949, **50**, 240.
3) Motokawa and Iwama, Tohoku J. Exp. Med., 1949, **50**, 292.
4) Motokawa and Iwama, Tohoku J. Exp. Med., 1949, **50**, 258.
5) Granit and Svætichin, Upsala Läkareförenings förhandlingar. Ny följd., 1939, **45**, 1.
6) Granit, Acta Physiol. Scand., 1942, **3**, 137.
7) Hartline, Amer. J. Physiol., 1938, **121**, 400.
8) Granit, J. Physiol., 1948, **107**, 54.
9) Granit, J. Neurophysiol., 1948, **11**, 239.
10) Granit, J. Neurophysiol., 1948, **11**, 253.
11) Donner, Experientia, 1949, **5**, 413.
12) Donner, Acta Physiol. Scand, 1950, **21**, Supp. 72.